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Signal Regulatory Protein α Regulates the Homeostasis of T Lymphocytes in the Spleen

Miho Sato-Hashimoto,* Yasuyuki Saito,*† Hiroshi Ohnishi,* Hiroko Iwamura,* Yoshitake Kanazawa,* Tetsuya Kaneko,* Shinya Kusakari,* Takenori Kotani,* Munemasa Mori,* Yoji Murata,‡ Hideki Okazawa,‡ Carl F. Ware,§ Per-Arne Oldenborg,‡ Yoshihisa Nojima,‡ and Takashi Matozaki* ,‡

The molecular basis for formation of lymphoid follicle and its homeostasis in the secondary lymphoid organs remains unclear. Signal regulatory protein α (SIRPα), an Ig superfamily protein that is predominantly expressed in dendritic cells or macrophages, mediates cell–cell signaling by interacting with CD47, another Ig superfamily protein. In this study, we show that the size of the T cell zone as well as the number of CD4+ T cells were markedly reduced in the spleen of mice bearing a mutant (MT) SIRPα that lacks the cytoplasmic region compared with those of wild-type mice. In addition, the expression of CCL19 and CCL21, as well as of IL-7, which are thought to be important for development or homeostasis of the T cell zone, was markedly decreased in the spleen of SIRPα MT mice. CD47-deficient mice also manifested phenotypes similar to SIRPα MT mice. These data suggest that SIRPα as well as its ligand CD47 are thus essential for steady-state homeostasis of T cells in the spleen. The Journal of Immunology, 2011, 187: 291–297.

Secondary lymphoid organs, spleen and lymph nodes (LN), are sites for induction of primary immune responses that provide critical microenvironments to facilitate interactions between cells of the innate and adaptive immune systems (1). In mouse spleen, the splenic white pulp consists of T cells zones surrounding a central arteriole, as well as B cell follicles and their surrounding marginal zones (1, 2). The positioning and segregation of T and B lymphocytes into their compartments in the white pulp in the spleen are controlled by homeostatic chemokines, including CCL19, CCL21, and CXCL13. CCL19 and CCL21 are thought to attract naive T cells that express CCR7, a receptor for CCL19 or CCL21, into periairial lymphoid sheathes (PALS) in the spleen (1, 3). By contrast, CXCL13 is thought to attract B cells, which express a CXCL13 receptor, CXCR5, into lymphoid follicles (1, 2, 4). Both CCL19 and CCL21 are produced by stromal cells in the PALS of spleen, named fibroblastic reticular cells (FRCs), whereas CXCL13 is produced by stromal cells present in the B cell follicles of the spleen (1, 3, 5). IL-7, which is also produced by FRCs in the T cell zone of the spleen, is thought to be a major cytokine that maintains the homeostasis of T cells, particularly naive T, by promoting their survival and proliferation (6).

In addition to these chemokines or cytokines, membrane-bound molecules are also thought to be important for homeostatic regulation of T cells. Indeed, interaction of self-Ag–presenting MHC molecules of dendritic cells (DCs) with TCR promotes the survival of naive T cells by activation of TCR signaling (6–9). Lymphotixin (LT) α1β2, a membrane-anchored heterotrimer expressed in B and T cells, interacts with the LTβ receptor (LTβR) expressed on FRCs and is essential for production of homeostatic chemokines, CCL19 or CCL21, by FRCs (2, 10, 11). However, the molecular basis for regulation of T cell homeostasis in the spleen by membrane-bound molecules remains largely uncharacterized. Signal regulatory protein α (SIRPα), also known as Src homology 2 domain-containing protein tyrosine phosphatase (SHP) substrate-1 or brain Ig-like molecule with tyrosine-based activation motifs (12, 13), is a transmembrane protein in which the extracellular region comprises three Ig-like domains and the cytoplasmic region contains ITIM that mediate binding of the protein tyrosine phosphatases SHP-1 and SHP-2. Tyrosine phosphorylation of SIRPα is triggered by various growth factors and cytokines as well as by integrin-mediated cell adhesion to extracellular...
matrix proteins. SIRPα is especially abundant in DCs or macrophages, whereas it is barely detectable in T or B lymphocytes (13–17). The extracellular region of SIRPα interacts with the ligand CD47, which is also a member of the Ig superfamily (12, 13, 18). In contrast to the relatively restricted distribution of SIRPα, CD47 is expressed in most cell types including a variety of hematopoietic cells (18).

SIRPα and CD47 constitute a cell–cell communication system, and such interaction plays important roles in both hematopoietic and immunological regulation. The interaction of CD47 on hematopoietic cells to SIRPα on macrophages is thought to prevent phagocytosis through an SIRPα-dependent activation of SHP-1 (13, 15, 19, 20). The SIRPα activation of SHP-1 determines both the life span of individual RBCs and the number of these cells in the circulation (13, 15, 19, 20). The SIRPα–CD47 interaction is also implicated in prevention of the clearance by splenic macrophages of transfused platelets or lymphocytes from the bloodstream (21–23), as well as in regulation of the ability of splenic macrophages to discriminate between viable and apoptotic cells (24). Moreover, the interaction of SIRPα in DCs with CD47 expressed in either hematopoietic or nonhematopoietic cells (such as stromal cells) is also important for homeostasis of conventional DCs (cDCs), particularly CD8– cDCs in secondary lymphoid organs through the regulation of the survival of cDCs (25–27).

Although the expression of SIRPα is minimal in T or B cells in the mouse spleen (as shown in Supplementary Fig. 1), we show that the size of T cell zone, as well as the number of CD4+ T cells in the spleen are markedly decreased in mice bearing a mutant (MT) version of SIRPα or CD47-deficient (CD47 knockout [KO]) mice. SIRPα and CD47 are thus important for the homeostasis of T cells in the spleen.

Materials and Methods

**Abs and reagents**

An agonistic rat mAb to mouse LTβR (4H8) was described previously (28, 29). A rat mAb to mouse CD16/32 (2.4G2) was isolated from the culture supernatant of hybridoma cells (kindly provided by C. J. Lagenaur, University of Pittsburgh, Pittsburgh, PA) was purified from culture supernatants of hybridoma cells. The mAbs were conjugated to sulfo-NHS-LC biotin [sulfosuccinimidyl-6-(biotinamido) hexanoate: Pierce]. Goat anti-mouse C2CL2, C1L9, and CXC1L3 polyclonal Abs were purchased from R&D Systems. Cy3-conjugated donkey Abs to goat IgG and hamster IgG were from Jackson ImmunoResearch Laboratories. FITC-conjugated mAbs to mouse B220 (RA3-6B2) and CD4 (L3T4), biotin-conjugated mAbs to mouse Thy1.2 (30-H12) and CD19 (eBio1D3), and a hamster mAb to mouse gp38 (ebio8.1.1) were from eBioscience. FITC-conjugated mAbs to CD8α (53-6.7), an allogeneic cytotoxic conjugated mAb to CD11c, biotin-conjugated mAbs to CD4 (RM4-5), and FITC- or PE-conjugated streptavidin were obtained from BD Bioscience. Allophycocyanin-Cy7–conjugated mAb to mouse CD11c, and biotin-conjugated streptavidin, allophycocyanin-conjugated mAb to CD11c, and allogeneic cytotoxic conjugated mAb to mouse CD8, PE-conjugated streptavidin, allophycocyanin-conjugated mAb to CD11c, and allogeneic cytotoxic conjugated mAb to mouse B220. The cells were washed again before suspension in the presence of propidium iodide to identify dead cells and analyzed by flow cytometry with the use of an FACScanto II or FACSAria II instrument (BD Biosciences) and FlowJo software (Tree Star).

**Preparation of cDNA and quantitative real-time PCR**

Total RNA was extracted from the freshly isolated spleen using Qiazol and the RNasey mini kit (Qiagen) 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) in 96-well plates (Roche Diagnostics). The amplification results were analyzed by the use of LightCycler 480 software (Roche Applied Science) and then normalized with GAPDH levels for each sample. Primer sequences for quantitative real-time PCR were as follows:

**Ccl2**, forward: 5’-ATCCGGCGCAATCTGTTTC-3’, reverse: 5’-GCGG-CCTTTGTTTCTCCTGGG-3’. 
**Ccl19**, forward: 5’-GGCCACGGTATTCTGGAAGC-3’, reverse: 5’-GGCCGTA-CTTTGACATCCGATTCTA-3’. 
**Ccl21**, forward: 5’-GGGGGTCTGATAATGCGGAA-3’, reverse: 5’-CTTTGATGTGTTGGTGAACACAACA-3’. 
**Ccl11**, forward: 5’-GGGCCAGGATTTCTGGAAGC-3’, reverse: 5’-GGCCGTA-CTTTGACATCCGATTCTA-3’. 
**Ccl13**, forward: 5’-GGGGTCTGATAATGCGGAA-3’, reverse: 5’-CTTTGATGTGTTGGTGAACACAACA-3’. 
**Ccl4**, forward: 5’-GGCCGTA-CTTTGACATCCGATTCTA-3’, reverse: 5’-GGCCGTA-CTTTGACATCCGATTCTA-3’. 
**Ltb**, forward: 5’-GGGGGTCTGATAATGCGGAA-3’, reverse: 5’-CTTTGATGTGTTGGTGAACACAACA-3’. 
**Gapdh**, forward: 5’-CTTTGATGTGTTGGTGAACACAACA-3’, reverse: 5’-CTTTGATGTGTTGGTGAACACAACA-3’. 

**Histological and immunohistochemical analyses of the spleen or peripheral LN**

For histological analysis, the spleen or peripheral LN (pLN) were removed and immediately fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Paraffin-embedded sections (4 μm) were stained with Mayer’s H&E. For immunohistofluorescence analysis, the spleens were directly embedded in optimal cutting temperature compound (Sakura) and immediately frozen in liquid nitrogen, followed by cryo-cutting into 8-μm sections and fixation in 4% paraformaldehyde in 0.1 M phosphate buffer. All sections were incubated for 1 h at room temperature in blocking solution (PBS with 5% BSA) and then stained with primary Abs diluted in PBS containing 5% BSA and 0.3% Triton X-100 overnight at 4°C. They were then washed with PBS, stained with Cy3- or FITC- conjugated Abs diluted in PBS containing 1% BSA and 0.3% Triton X-100 for 1 h at room temperature, and they were washed again with PBS. Fluorescence or bright-field images were acquired with a BX-51 microscope (Olympus), a color cooled CCD camera (DP71; Olympus), and DP controller software (Olympus). For measurement of areas for the T zone and B zone or of gp38-positive regions in the spleen, cross-sections were made through central segments of the spleen, stained for Thy1.2 and B220 and gp38, and images were then acquired at ×4 (T zone and B zone) or ×10 (gp38) objective magnification, respectively. By the use of Image J software (National Institutes of Health), the values for positively stained regions in a microscopic image of the spleen from each mouse were obtained and averaged.

**Cell preparation and flow cytometry**

Cell suspensions were prepared from spleen or pLN as described previously (19, 25). For preparation of allogeneic or syngeneic LN cells, the spleen or pLN were minced and then digested with collagenase (Wako) at 400 U/ml in the presence of 5 mM EDTA for 30 min at 37°C. The undigested fibrous material was removed by filtration through a 70-μm cell strainer (BD Falcon), and RBCs in the filtrate were lysed with Gey’s solution. The remaining cells were washed twice with PBS and then subjected to flow cytometric analysis. The cells were first incubated with an mAb to mouse CD16/32 to prevent nonspecific binding of labeled Abs to Fcγ receptors and then stained with a biotin-conjugated mAb to mouse CD4. The cells were washed and incubated with an FITC-conjugated mAb to mouse CD8, PE-conjugated streptavidin, allophycocyanin-conjugated mAb to CD11c, and allogeneic cytotoxic conjugated mAb to B220. The cells were washed again before suspension in the presence of propidium iodide to identify dead cells and analyzed by flow cytometry with the use of an FACScanto II or FACSAria II instrument (BD Biosciences) and FlowJo software (Tree Star).

**Animal experiment**

Mice that express an MT version of SIRPα lacking most of the cytoplasmic region were described previously (30, 31). The mice were backcrossed onto the C57BL/6 background for five generations. CD47 KO mice were described previously (19, 25) and backcrossed to the C57BL/6 background for >10 generations. Sex- and age-matched mice at 6–12 wk of age were studied. Mice were bred and maintained in the Institute of Experimental Animal Research of Gunma University under specific pathogen-free conditions, and care and use were in accordance with the animal care guidelines of Gunma University.
Bone marrow chimeras
Recipient wild-type (WT) or MT mice were subjected to lethal irradiation (9.5 Gy) and then injected i.v. with 5 × 10^6 bone marrow (BM) cells obtained from either WT or MT donor mice as described previously (25). Six to 8 wk after BM transplantation, the recipient mice were killed, and the spleen was subjected for histology, immunohistofluorescence analysis, and extraction of RNA for real-time PCR.

Isolation of T and B cells
The spleen was gently ground with sterilized frosted slide glasses in PBS followed by filtration through a 70-μm cell strainer (BD falcon) to remove fibrous components. RBCs were lysed with Gey’s solution and washed twice in PBS. For isolation of T cells, splenocyte suspension was filtrated again through nylon wool before purification. T or B cells were purified with the use of magnetic beads coated with a biotin-conjugated mAb to Thy1.2 or CD19, respectively, and an MACS column (Miltenyi Biotec). The purity of the isolated Thy1.2+ T cells or CD19+ B cells were >90% as determined by flow cytometry.

Analysis of in vivo gene expression by the injection of agonistic mAbs to LTβR
In vivo injection of agonistic mAb to LTβR was performed as described previously with minor modifications (28). In brief, WT or MT mice were injected i.p. either with 50 μg control rat IgG Ab or 50 μg agonistic anti-LTβR mAbs. Twenty-four hours later, spleen was isolated and cDNA was prepared, and then mRNA expression of CCL21 (Ccl21) or NFκb2 p100 (Nfk2b) was analyzed by real-time PCR.

Statistical analysis
Data are presented as means ± SE and were analyzed by Student t test or by one-way ANOVA, and post hoc comparisons were made using the Tukey-Kramer test with use of Stat View 5.0 software (SAS Institute). A p value <0.05 was considered statistically significant.

Results
Impairment of T cell zone development in the spleen of SIRPα MT mice
We previously showed that the SIRPα MT mice displayed mild splenomegaly characterized by expansion of the red pulp (15) (Fig. 1A). The expanded red pulp of the spleen was attributable to an increase in erythropoiesis to compensate for persistent anemia caused by increased phagocytic clearance of RBCs. The MT SIRPα protein expressed in the transgenic mice fails to undergo tyrosine phosphorylation or form a complex with SHP-1 or SHP-2 (20). Given the importance of the cytoplasmic region of SIRPα for signaling by this protein, the function of SIRPα is thought to be eliminated in the MT mice (15, 20). In contrast to the expanded red pulp, the white pulp in SIRPα MT mice was significantly smaller and more segmented when compared with WT mice (Fig. 1A). Quantitative immunohistofluorescence analyses of T cells (Thy1.2+) and B cells (B220+) in the spleens of SIRPα MT mice revealed the area of the white pulp was markedly reduced, particularly in the T cell zone around the arteriole (Fig. 1B, 1C). We consistently observed that the number of CD4+ T cells in the spleen of SIRPα MT mice was markedly decreased compared with that of WT mice (Fig. 1D). The number of CD8+ T cells was slightly decreased in the spleen of SIRPα MT mice, although such decrease was not statistically significant. By contrast, the number of B cells in the spleen did not differ between WT and SIRPα MT mice (Fig. 1D). The size of T cell zones as well as the absolute number of CD4+ or CD8+ T cells in pLNs did not differ between WT and SIRPα MT mice (Supplemental Fig. 2). The proportions of CD4+ T cells as well as CD8+ T cells and B cells in the peripheral blood of MT mice were also similar to those apparent for WT mice (Supplemental Fig. 3). These results suggest that the alteration of the T cell zone is specific to the spleen of SIRPα MT mice.

Reduced expression of CCL-19, CCL-21, and IL-7 in the spleen of SIRPα MT mice
The smaller size of the T cell zone as well as the reduced number of CD4+ T cells in the spleen of SIRPα MT mice suggested that homing or survival of T cells is impaired in the MT mice. Expression levels of CCL19, CCL21, and CXCL13 mRNA in the spleen of SIRPα MT mice were markedly decreased in MT mice compared with WT mice (Fig. 2A). Immunohistofluorescence analysis showed a loss of CCL19 and CCL21 in the T cell zone of SIRPα MT mice, whereas CXCL13 in the B cell follicles appeared the same as in WT mice (Fig. 2C). IL-7 is important for the survival and homeostasis of T cells (6). We found that IL-7 mRNA expression in the spleen of SIRPα MT mice was also markedly decreased compared with that of WT mice (Fig. 2B). Thus, both recruitment and survival of T cell may be compromised in SIRPα MT mice.

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**FIGURE 1.** Impairment of T cell zone development in the spleen of SIRPα MT mice. A, Paraffin sections of the spleen from WT or SIRPα MT mice were stained with H&E. The boxed regions of the left panels are shown at higher magnification in the right panels. Scale bars, 500 μm (left panels) and 200 μm (right panels). B, Frozen sections of the spleen from WT or MT mice were double-stained with an mAb to B220 (green) and an mAb to Thy1.2 (red). Scale bar, 500 μm. C, By the use of Image J software (National Institutes of Health), the areas for Thy1.2-positive T cell zones or B220-positive B cell zones in the spleen sections prepared as in B were measured per each image. Data are means ± SE for four mice per group and representative of three independent experiments. **p < 0.01 (Student t test).**
The expression of CCL19, CCL21, and CXCL13 and IL-7 mRNA in the spleen of SIRPα MT mice did not differ between WT and SIRPα KO mice (Fig. 3A). Reduced expression of CCL19, CCL21, and IL-7 in the spleen of SIRPα MT mice was evaluated by quantitative PCR. The level of expression of each mRNA was normalized to that of GAPDH mRNA and presented as fold increase relative to the value for WT mice. Data are means ± SE for a total of 10 mice per group in three independent experiments. *p < 0.05, **p < 0.01 (Student’s t test).

Importance of CD47 for development of the splenic T cell zone

We next examined the role of CD47 in the regulation of T cell homeostasis in the spleen. The area of the white pulp in spleens of CD47 KO mice was smaller and segmented, indicating a phenocopy of the SIRPα MT mice (Fig. 3A). Consistently, CD47 KO mice showed a loss in the area of the T cell zone in the spleen (Fig. 3B) and decreased numbers of CD4+ T cells (Fig. 3C) and the expression of CCL19, CCL21, and CXCL13 and IL-7 mRNA (Fig. 3D, 3E).

Importance of hematopoietic SIRPα for development of the splenic T cell zone

Among hematopoietic cells, SIRPα is especially abundant in DCs and macrophages (13), whereas it is barely detectable in T or B lymphocytes, as shown in Supplemental Fig. 1. In addition, the expression of SIRPα is low in nonhematopoietic cells such as fibroblasts and endothelial cells (30, 34). We thus examined whether SIRPα expression is required in hematopoietic or nonhematopoietic tissues for the development of the T cell zone in the spleen. BM chimeras established in irradiated WT mice were reconstituted with BM from SIRPα MT (MT→WT) or as control WT (WT→WT) mice. The resulting chimeric mice displayed a marked decrease in the T cell zone in the spleen in MT→WT chimeras compared with those in WT→WT chimeras (Fig. 4A). The area of the B cell follicles was slightly but not significantly reduced in the spleen of MT→WT chimeras compared with that of WT→WT chimeras (Fig. 4A). By contrast, the area of the T cell zone, as well as that for B cell follicles, in the spleen was similar.
Reduced expression of LTα, LTβ, or LTβR and impaired LT signaling in the spleen of SIRPα MT mice

The interaction of LTαβ on B or T cells or that of LIGHT (TNFSF14) on T cells with the LTβR expressed on FRCs is thought to be important for homeostatic regulation of T cells by promotion of CCL19 or CCL21 production from FRCs (10, 11). To investigate the cause of the poor development of the T cell zone, as well as the reduction of CCL19 or CCL21, in the spleen of SIRPα MT mice, we next evaluated the mRNA expression levels of LTα, LTβ, LIGHT, and LTβR in the spleen of the MT mice. We found that the mRNA expression levels of LTα, LTβ, and LIGHT, as well as that of LTβR, in the spleen of SIRPα MT mice were markedly decreased compared with WT mice (Fig. 5A). However, the expression level of either LTα or LTβ in isolated T cells or B cells from SIRPα MT mice did not differ from that of WT mice (Fig. 5B).

We next examined whether the LTβR-mediated signaling was impaired in SIRPα MT mice in vivo. Activation by LT binding of LTβR on stromal cells promotes stimulation of the canonical pathway of NF-κB, resulting in the transcription of VCAM-1 as well as p100, a precursor of NF-κB2 (p52) (10, 11, 28). In addition, the activation of LTβR also promotes the processing of

FIGURE 4. Importance of hematopoietic SIRPα for development of the splenic T cell zone. A, WT or SIRPα MT mice were lethally irradiated and then reconstituted with 5 × 10⁶ BM cells from WT or MT mice for generating WT→WT, WT→MT, or MT→WT chimeras. Eight weeks after transplantation, spleens were harvested, and frozen sections of the spleen from each chimera were stained with mAbs to B220 (green) and to Thy1.2 (red). Scale bar, 500 μm (upper panels). The area for Thy1.2-positive T cell zone or B220-positive B cell zone was measured per each image by the use of Image J software (National Institutes of Health). Data are means ± SE of eight to nine mice per group in two independent experiments (lower panels). Expression of CCL19 (Ccl19), CCL21 (Ccl21) or CXCL13 (Ccl13) mRNA (B) or IL-7 (Il7) mRNA (C) in the spleen of WT→WT, WT→MT, or MT→WT chimeras was evaluated by quantitative PCR. The level of expression of each mRNA was normalized to that of GAPDH mRNA and presented as fold increase relative to the value for WT→WT chimeras. Data are means ± SE of four mice per group and are representative in two independent experiments. *p < 0.05, **p < 0.01 (one-way ANOVA, followed by the Tukey-Kramer test).

in the WT→MT and WT→WT chimeras (Fig. 4A). These results indicate that SIRPα expression in the hematopoietic compartment is required for proper formation of the T cell zone in the spleen. SIRPα MT mice displayed mild splenomegaly characterized by expansion of the red pulp as described above (Fig. 1A). We observed the marked expansion of red pulp in the spleen of SIRPα MT→WT chimeras and mild or slight expansion in WT→SIRPα MT chimeras (Supplemental Fig. 4).

The mRNA expression levels of CCL19, CCL21, and CXCL13 were markedly decreased in the spleen of MT→WT chimeras compared with those in WT→WT chimeras (Fig. 4B). In addition, the mRNA expression levels of CCL19 and CCL21, but not that of CXCL13, were also decreased in WT→MT chimeras (Fig. 4B). Furthermore, IL-7 mRNA expression was also decreased in the spleen of MT→WT chimeras, but not in WT→MT chimeras (Fig. 4C).
p100 to p52 NF-κB2, a noncanonical pathway of NF-κB (10, 11, 28). In association with RelB, p52 NF-κB2 thereafter translocates to the nucleus, resulting in the transcription of CCL21, CCL19, or CXCL13. Expression of CCL21 mRNA in the spleen of WT mice was markedly increased by an agonistic mAb to LTβR 24 h after injection as described previously (28, 29) (Fig. 5C). By contrast, such increase of CCL21 mRNA expression was not apparent in the spleen of SIRPα MT mice (Fig. 5C). Injection of mAbs to LTβR also increased the mRNA expression of p100 in the spleen of WT mice as described previously (28, 29), whereas it had no effect on CCL21 or p100 mRNA levels in SIRPα MT mice (Fig. 5C).

**Discussion**

We demonstrate in this study that the size of T cell zone and the number of resident CD4+ T cells in the spleen were markedly reduced in the spleen of SIRPα MT mice. In addition, the mRNA expression of CCL19, CCL21, and IL-7 or the protein expression of the former two chemokines was also decreased in the spleen of SIRPα MT mice. Given that CCL19 and CCL21 as well as IL-7 are thought to be important for organization and maintenance of the T cell zone (1, 3, 6, 11), the reduction in the size of the T cell zone and CD4+ T cell number in the spleen of SIRPα MT mice is, at least in part, attributable to the decreased expression of CCL19, CCL21, or IL-7. Both CCL19 and CCL21, as well as IL-7, are produced by stromal cell FRCs in the PALS of spleen. Indeed, the immune reactivity of gp38, a marker for FRCs, was markedly decreased in the spleen of SIRPα MT mice, suggesting that the decrease of the cell population of FRCs might be a cause for the reduced expression of CCL19, CCL21, or IL-7 in the spleen of SIRPα MT mice.

The expression of SIRPα is minimal in T cells as shown in Supplemental Fig. 1, suggesting that SIRPα is unlikely required in a cell-autonomous manner for homeostatic regulation of T cells in the spleen. However, the reduction of the T cell zone as well as the decreased mRNA expression of CCL19 or CCL21 and IL-7 in the spleen were observed in hematopoietic BM chimeras. We indeed found that the mRNA expression levels of LTα, LTβ, and LIGHT were markedly decreased in the spleen of SIRPα MT mice. LTα, LTβ, and LIGHT are thought to be important for homeostatic regulation of T cells by producing CCL19 or CCL21 from FRCs. Indeed, the spleens of adult LTα−/−, LTβ−/−, and LTβR−/− mice showed marked defects in the development of white pulps in the spleen (2, 35–37). LTα and LTβ are predominantly expressed on B cells or T cells (11, 38), whereas LIGHT is expressed in T cells, DCs, or NK cells (39). Thus, these results suggest that hematopoietic SIRPα is, at least in part, important for homeostatic regulation of T cells or expression of CCL19 or CCL21 in the spleen through the action of LTβ or LIGHT. Of interest is that the expression level of either LTα or LTβ in isolated T cells or B cells from SIRPα MT mice did not differ from that of WT mice. Thus, the reduction of LTα and LTβ in the whole spleen of SIRPα MT mice is, at least in part, attributable to the reduction of T cell population. However, LTα and LTβ are also expressed in lymphoid tissue inducer (LTi) cells (40) or NK cells (38). Moreover, expression of CCL21 or of CCL19 was reduced in the secondary lymphoid organs of LTβ KO mice but not in B cell-specific LTβ KO or T and B cell-specific LTβ KO mice, suggesting that expression of CCL21 or CCL19 is largely dependent on LT produced by T or B lymphocytes (41). Thus, SIRPα is required for expression of LTα or LTβ that is produced by yet-unidentified hematopoietic cells such as LTi cells, which might express SIRPα. By the use of BM chimera, we found that nonhematopoietic SIRPα is also required for expression of CCL21 or CCL19 in the spleen. Given the expression of SIRPα in nonhematopoietic cells such as fibroblasts and endothelial cells (30, 34), SIRPα is potentially expressed in FRCs in the spleen and thus required for the homeostatic regulation of FRCs.

In addition to the reduction of LTα or LTβ mRNA expression in the spleen, we showed that the effect of the agonistic mAb to LTβR on mRNA expression of CCL21 or p100 was markedly reduced in the spleen of SIRPα MT mice. Such reduced response is presumably attributable to the reduction of LTβR mRNA expression in the spleen of SIRPα MT mice. Furthermore, SIRPα might be important for the signaling pathway downstream of LTβR, and impairment of LTβR signaling thus participates in the reduced expression of homeostatic chemokines CCL21 or CCL19 in the spleen of SIRPα MT mice.

SIRPα MT mice manifest mild splenomegaly that is likely attributable to the increased number of RBCs in the spleen (15). Thus, a decrease of the white pulp of SIRPα MT mice might be a secondary effect of the splenomegaly. However, we demonstrated in this study that the number of CD4+ T cells, but not that of B cells, was indeed reduced in the spleen of SIRPα MT mice. Moreover, CD47 KO mice also manifested the reduction of T cell zone and the decreased cell number of CD4+ T cells, whereas they did not manifest splenomegaly (M. Saito-Hashimoto, Y. Saito, and T. Matozaki, unpublished observations). Thus, the reduced size of white pulp is unlikely attributable to a secondary effect of the splenomegaly in SIRPα MT mice. By contrast, the number of CD11c−/− DCs (cDCs) is markedly reduced in the spleen of SIRPα MT mice (25). Given that interaction of self-Ag–presenting MHC molecules of DCs with TCR promotes the survival of naive T cells (6), the reduction of cDCs in the spleen of SIRPα MT mice might participate in the decrease in CD4+ T cells.

The reason why the phenotypes of MT mice are specific to the spleen is currently unknown. However, such tissue-specific difference was noted in some gene-KO mice. BCR-deficient mice manifested a marked decrease of T cells as well as of CCL21 in the spleen but not in pLNs (42). In addition, CD30-deficient mice showed impaired B/T segregation in the spleen but not in pLNs (43), and the authors suggested the importance of LTi cells in regulation by CD30 of B/T segregation. We indeed measured the mRNA expression of CCL21, CCL19, CXCL13, and IL-7 in the pLNs. Expression levels of mRNA for these chemokines or a cytokine in the pLNs did not differ between WT and SIRPα MT mice, again suggesting that the phenotypes of SIRPα MT mice are specific to the spleen. Thus, SIRPα is likely an element that regulates T cell homeostasis differentially in the spleen and pLNs.

Similar to SIRPα MT mice, CD47 KO mice manifested the marked reduction of the T cell zone and the decreased cell number of CD4+ T cells, as well as reduced expression of CCL19 and CCL21, CXCL13, or IL-7 in the spleen. Given that CD47 is a ligand for SIRPα, the similarity of these CD47 KO phenotypes to those of SIRPα MT mice suggests that CD47 and SIRPα form a pathway that regulates splenic T cell microenvironment. In contrast to SIRPα, however, we have not determined yet whether hematopoietic or nonhematopoietic CD47 is important for such regulation. With regard to the role of hematopoietic CD47, trans interaction of CD47 on T cells with hematopoietic SIRPα might be important. With regard to the role of nonhematopoietic CD47, trans interaction of CD47 on nonhematopoietic cells, such as stromal cells or endothelial cells, with hematopoietic SIRPα might be important for the homeostatic regulation of T cells in the spleen. Indeed, CD47 is expressed in splenic and BM stromal cells (44) (Y. Saito and T. Matozaki, unpublished observations) as well as in endothelial cells (18).
Over our present study provides a new insight into the molecular basis for regulation of T cell homeostasis in the spleen by membrane-bound molecules. Further study is clearly necessary to understand the detailed mechanism by which SIRPα that is not expressed in T cells regulates T cell homeostasis by interacting CD47.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Figure 1. Minimal expression of SIRPα on CD8+ T, CD4+ T, and B cells. Splenocytes isolated from WT mice were incubated with a biotin-conjugated mAb to SIRPα or an isotype-matched control mAb. The cells were also stained with an FITC-conjugated mAb to CD8, PE-conjugated streptavidin, a PE-Cy7–conjugated mAb to CD4, an APC-conjugated mAb to CD11c, an APC-Cy7–conjugated mAb to B220, and PI. The expression of SIRPα (open traces) or staining with the isotype control (filled traces) were analyzed for CD4+ T cells, CD8+ T cells, B cells (defined as CD11c− CD4+ cells, CD11c− CD8+ cells, or CD11c− B220+ cells) or CD11c+ DCs, respectively, among all viable cells by six-color flow cytometry. Data are representative of three separate experiments.

Supplementary Figure 2. Normal development of T cell zone in the peripheral LNs of SIRPα MT mice. A, Frozen sections of the peripheral LNs (pLNs) from WT or MT mice were double-stained with a mAb to B220 (green) and a mAb to Thy1.2 (red) (left panels). Scale bar, 500 μm. B, The absolute numbers of B cells (B220+), CD4+ T cells (CD4+) and CD8+ T cells (CD8+) in the pLNs of WT or MT mice were determined by flow cytometry (right panel). Data are means ± SE for a total of three mice per group in three independent experiments (right panel). C, Expression of CCL19 (Ccl19), CCL21 (Ccl21), CXCL13 (Cxcl13) or IL-7 (Il7) mRNA in pLN of WT or SIRPα MT mice was evaluated by quantitative PCR. The level of expression of each mRNA was normalized to that of GAPDH mRNA and presented as fold increase relative to the value for WT mice. Data were means ± SE for a total of five mice per group and were representative from two independent experiments.

Supplementary Figure 3. Proportions of lymphocytes in the peripheral blood of WT and SIRPα MT mice. The percentages of B cells, CD4+ T cells and CD8+ T cells among total cells in the peripheral blood of WT or SIRPα MT mice were determined by
flow cytometry. Data are means ± SE for a total of three mice per group and are representative from two independent experiments.

**Supplementary Figure 4. Histological analysis of the spleen of BM chimera.** WT or SIRPα MT mice were lethally irradiated and then reconstituted with $5 \times 10^6$ BM cells from WT or MT mice for generating WT $\rightarrow$ WT, MT $\rightarrow$ WT or WT $\rightarrow$ MT chimeras. Eight weeks after transplantation, spleens were harvested and paraffin sections of the spleen from each chimera were stained with H&E. Each micrograph was representative of 8 animals per group and was from two separate experiments. Scale bar, 500 μm.
Supplementary Figure 3
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Supplementary Figure 4
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