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

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Abstract

The molecular basis for formation of lymphoid follicle and its homeostasis in the secondary lymphoid organs remains unclear. Signal regulatory protein α (SIRP α), 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) SIRP α 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 SIRP α MT mice. By the use of bone marrow chimera, we found that hematopoietic SIRP α is important for development of the T cell zone as well as the expression of CCL19 and CCL21 in the spleen. The expression of lymphotoxin and its receptor, lymphotoxin β receptor, as well as the *in vivo* response to lymphotoxin β receptor stimulation were also decreased in the spleen of SIRP α MT mice. CD47-deficient mice also manifested phenotypes similar to SIRP α MT mice. These data suggest that SIRP α as well as its ligand CD47 are thus essential for steady-state homeostasis of T cells in the spleen.

Secondary lymphoid organs, spleen and lymph nodes (LN), are sites for induction of primary immune responses that provide critical microenvironments to facilitate interactions

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Disclosures

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between cells of the innate and adaptive immune systems (1). In mouse spleen, the splenic white pulp consists of T cell 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 periarterial 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 cells, 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). Lymphotoxin (LT) $\alpha_1\beta_2$, a membrane-anchored heterotrimer expressed in B and T cells, interacts with the LT β receptor (LT β R) 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 (SIRP α), 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 SIRP α is triggered by various growth factors and cytokines as well as by integrin-mediated cell adhesion to extracellular matrix proteins. SIRP α is especially abundant in DCs or macrophages, whereas it is barely detectable in T or B lymphocytes (13–17). The extracellular region of SIRP α 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 SIRP α , CD47 is expressed in most cell types including a variety of hematopoietic cells (18).

SIRP α 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 SIRP α on macrophages is thought to prevent phagocytosis through an SIRP α -dependent activation of SHP-1 (13, 15, 19, 20). The SIRP α 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 SIRP α –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 macrophages to discriminate between viable and apoptotic cells (24). Moreover, the interaction of SIRP α 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 SIRP α is minimal in T or B cells in the mouse spleen (as shown in Supplemental 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 SIRP α or CD47-deficient (CD47 knockout [KO]) mice. SIRP α 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 LT β R (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 K. Okumura, Juntendo University, Tokyo, Japan). A rat mAb to mouse SIRP α (kindly provided by C.F. 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 CCL21, CCL19, and CXCL13 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 CD8 α (53-6.7), an allophycocyanin-conjugated mAb to CD11c, biotin-conjugated mAbs to CD4 (RM4-5), and FITC- or PE-conjugated streptavidin were obtained from BD Biosciences. Allophycocyanin-Cy7-conjugated mAb to B220 (RA3-6B2) were from Bio-Legend (San Diego, CA). Anti-biotin microbeads were from Miltenyi Biotec (Bergisch Gladbach, Germany). RPMI 1640 medium (Sigma-Aldrich) was supplemented with 10% heat-inactivated FBS, 50 μ M 2-ME, 2 mM L-glutamine, 10 mM HEPES-NaOH (pH 7.4), penicillin (100 U/ml), streptomycin (100 μ g/ml), and 1 mM sodium pyruvate to yield complete medium.

Animals

Mice that express an MT version of SIRP α 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.

Histological and immunohistofluorescence analyses of the spleen or peripheral LNs

For histological analysis, the spleen or peripheral LNs (pLNs) were removed and immediately fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Paraffin-embedded sections (4 μ m) 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 cutting into 8- μ m 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 or gp38, and images were then acquired at $\times 4$ (T zone and B zone) or $\times 10$ (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 pLNs as described previously (25). For preparation of splenocytes or pLN cells, the spleen or pLNs 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 mAbs 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 allophycocyanin-Cy7-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).

Preparation of cDNA and quantitative real-time PCR

Total RNA was extracted from the freshly isolated spleen using Qiazol and the RNeasy 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: *Ccl21*, forward: 5'-ATCCCGGCAATCCTGTTCTC-3', reverse: 5'-GGG-GCTTTGTTTCCCTGGG-3'; *Ccl19*, forward: 5'-GGGGTGCTAATGAT-GCGGAA-3', reverse: 5'-CCTTAGTGTGGTGAACACAACA-3'; *Cxcl13*, forward: 5'-GGCCACGGTATTCTGGAAGC-3', reverse: 5'-GGGCGTA-
ACTTGAATCCGATCTA-3'; *Lta*, forward: 5'-TCCACTCCCTCAGAA-GCACT-3', reverse: 5'-AGAGAAGCCATGTCGGAGAA-3'; *Ltb*, forward: 5'-TGCGGATTCTACACCAGATCC-3', reverse: 5'-ACTCATCCAAGC-GCCTATGA-3'; *Tnfrsf14* (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'-CAACCCAGCAG-CACATCTTA-3', reverse: 5'-ATACGTCAAGCCCCTCAAGA-3'; *LtbR*, forward: 5'-AGCCGAGGTACAGATGAAAT-3', reverse: 5'-CCCTGG-
ATCTCACATCTGGT-3'; *Il7*, forward: 5'-GATAGTAATTGCCCCGAATA-
ATGAACCA-3', reverse: 5'-GTTTGTGTGCCTTGTGATACTGTTAG-3'; *Nfkb2* (p100), forward: 5'-CTGCTGCTAAATGCTGCTCA-3', reverse: 5'-
AGCAGTTGCTCCAGGTTCTG-3'; and *Gapdh*, forward: 5'-AGGTC-
GGTGTGAACGGATTTG-3', reverse: 5'-TGTAACCATGTAGTTGAG-GTCA-3'.

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, immunohistochemistry 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- μ m 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 LT β R

In vivo injection of agonistic mAb to LT β R was performed as described previously with minor modifications (28). In brief, WT or MT mice were injected i.p. either with 50 μ g control rat IgG Ab or 50 μ g agonistic anti-LT β R mAbs. Twenty-four hours later, spleen was isolated and cDNA was prepared, and then mRNA expression of CCL21 (*Ccl21*) or NF κ B2 p100 (*Nfkb2*) was analyzed by real-time PCR.

Statistical analysis

Data are presented as means $\pm$ 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 SIRP α MT mice

We previously showed that the SIRP α 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 SIRP α 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 SIRP α for signaling by this protein, the function of SIRP α is thought to be eliminated in the MT mice (15, 20). In contrast to the expanded red pulp, the white pulp in SIRP α 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 SIRP α 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 SIRP α 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 SIRP α 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 SIRP α 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 SIRP α 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 SIRP α MT mice.

Reduced expression of CCL-19, CCL-21, and IL-7 in the spleen of SIRP α MT mice

The smaller size of the T cell zone as well as the reduced number of CD4⁺ T cells in the spleen of SIRP α 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

SIRP α MT mice were markedly decreased in MT mice compared with WT mice (Fig. 2A). Immunohistochemistry analysis showed a loss of CCL19 and CCL21 in the T cell zone of SIRP α 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 SIRP α 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 SIRP α MT mice. FRCs in the T cell zone express CCL19, CCL21, and IL-7 (1, 6), suggesting a defect in the stromal cells in SIRP α MT mice. The expression of the mucin gp38, which identifies FRCs in the T cell zone (32, 33), was significantly suppressed, consistent with a stromal cell phenotype in SIRP α MT mice (Fig. 2D). Expression levels of mRNA for CCL19, CCL21, CXCL13, and IL-7 in the pLNs of SIRP α MT mice did not differ between WT and SIRP α MT mice (Supplemental Fig. 2).

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 pheno-copy of the SIRP α 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 SIRP α for development of the splenic T cell zone

Among hematopoietic cells, SIRP α 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 SIRP α is low in nonhematopoietic cells such as fibroblasts and endothelial cells (30, 34). We thus examined whether SIRP α expression is required in hematopoietic or non-hematopoietic tissues for the development of the T cell zone in the spleen. BM chimeras established in irradiated WT mice were reconstituted with BM from SIRP α MT (MT $\rightarrow$ WT) or as control WT (WT $\rightarrow$ WT) mice. The resulting chimeric mice displayed a marked decrease in the T cell zone in the spleen in MT $\rightarrow$ WT chimeras compared with those in WT $\rightarrow$ WT chimeras (Fig. 4A). The area of the B cell follicles was slightly but not significantly reduced in the spleen of MT $\rightarrow$ WT chimeras compared with that of WT $\rightarrow$ 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 in the WT $\rightarrow$ MT and WT $\rightarrow$ WT chimeras (Fig. 4A). These results indicate that SIRP α expression in the hematopoietic compartment is required for proper formation of the T cell zone in the spleen. SIRP α 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 SIRP α MT $\rightarrow$ WT chimeras and mild or slight expansion in WT $\rightarrow$ WT $\rightarrow$ SIRP α MT chimeras (Supplemental Fig. 4).

The mRNA expression levels of CCL19, CCL21, and CXCL13 were markedly decreased in the spleen of MT $\rightarrow$ WT chimeras compared with those in WT $\rightarrow$ WT chimeras (Fig. 4B). In addition, the mRNA expression levels of CCL19 and CCL21, but not that of CXCL13, were also decreased in WT $\rightarrow$ MT chimeras (Fig. 4B). Furthermore, IL-7 mRNA expression was also decreased in the spleen of MT $\rightarrow$ WT chimeras, but not in WT $\rightarrow$ MT chimeras (Fig. 4C).

Reduced expression of LT α , LT β , or LT β R and impaired LT signaling in the spleen of SIRP α MT mice

The interaction of LT $\alpha_1\beta_2$ on B or T cells or that of LIGHT (TNFSF14) on T cells with the LT β R 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 SIRP α MT mice, we next evaluated the mRNA expression levels of LT α , LT β , LIGHT, and LT β R in the spleen of the MT mice. We found that the mRNA expression levels of LT α , LT β , and LIGHT, as well as that of LT β R, in the spleen of SIRP α MT mice were markedly decreased compared with WT mice (Fig. 5A). However, the expression level of either LT α or LT β in isolated T cells or B cells from SIRP α MT mice did not differ from that of WT mice (Fig. 5B).

We next examined whether the LT β R-mediated signaling was impaired in SIRP α MT mice in vivo. Activation by LT binding of LT β R on stromal cells promotes stimulation of the canonical pathway of NF- κ B, resulting in the transcription of VCAM-1 as well as p100, a precursor of NF- κ B2 (p52) (10, 11, 28). In addition, the activation of LT β R also promotes the processing of p100 to p52 NF- κ B2, a noncanonical pathway of NF- κ B (10, 11, 28). In association with RelB, p52 NF- κ B2 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 LT β R 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 SIRP α MT mice (Fig. 5C). Injection of mAbs to LT β R 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 SIRP α 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 SIRP α 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 SIRP α 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 SIRP α MT mice is likely, 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 immunoreactivity of gp38, a marker for FRCs, was markedly decreased in the spleen of SIRP α 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 SIRP α MT mice.

The expression of SIRP α is minimal in T cells as shown in Supplemental Fig. 1, suggesting that SIRP α 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 LT α , LT β , and LIGHT were markedly decreased in the spleen of SIRP α MT mice. LT α , LT β , and LIGHT are thought to be important for homeostatic regulation of T cells by producing of CCL19 or CCL21 from FRCs. Indeed, the spleens of adult LT α ^{-/-}, LT β ^{-/-}, and LT β R^{-/-} mice showed marked defects in the development of white pulps in the spleen (2, 35–37). LT α and LT β 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 SIRP α 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 LT α β or LIGHT. Of interest is that the expression level of either LT α or LT β in isolated T cells or B cells from SIRP α MT mice did not differ from that of WT mice. Thus, the reduction of LT α and LT β in the whole spleen of SIRP α MT mice is, at least in part, attributable to the reduction of T

cell population. However, LT α and LT β 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 LT β KO mice but not in B cell-specific LT β KO or T and B cell-specific LT β KO mice, suggesting that expression of CCL21 or CCL19 is largely independent of LT produced by T or B lymphocytes (41). Thus, SIRP α is required for expression of LT α or LT β that is produced by yet-unidentified hematopoietic cells such as LTi cells, which might express SIRP α . By the use of BM chimera, we found that nonhematopoietic SIRP α is also required for expression of CCL21 or CCL19 in the spleen. Given the expression of SIRP α in non-hematopoietic cells such as fibroblasts and endothelial cells (30, 34), SIRP α is potentially expressed in FRCs in the spleen and thus required for the homeostatic regulation of FRCs.

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

SIRP α 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 SIRP α 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 SIRP α 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. Sato-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 SIRP α MT mice. By contrast, the number of CD11c^{high} DCs (cDCs) is markedly reduced in the spleen of SIRP α 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 SIRP α 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 SIRP α MT mice, again suggesting that the phenotypes of SIRP α MT mice are specific to the spleen. Thus, SIRP α is likely an element that regulates T cell homeostasis differentially in the spleen and pLNs.

Similar to SIRP α 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 SIRP α , the similarity of these CD47 KO phenotypes to those of SIRP α MT mice suggests that CD47 and SIRP α form a pathway that regulates splenic T cell microenvironment. In contrast to SIRP α , 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 SIRP α 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 SIRP α 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).

Overall, 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 SIRP α that is not expressed in T cells regulates T cell homeostasis by interacting CD47.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

BM	bone marrow
cDC	conventional dendritic cell
DC	dendritic cell
FRC	fibroblastic reticular cell
KO	knockout
LIGHT	lymphotoxin-like, exhibits inducible expression and competes with HSV glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes
LN	lymph node
LT	lymphotoxin
LTi	lymphoid tissue inducer
LTβR	lymphotoxin β receptor
MT	mutant
PALS	periarterial lymphoid sheath
pLN	peripheral lymph node
SHP	Src homology 2 domain-containing protein tyrosine phosphatase
SIRPα	signal regulatory protein α
WT	wild-type

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FIGURE 1.

Impairment of T cell zone development in the spleen of SIRP α MT mice. *A*, Paraffin sections of the spleen from WT or SIRP α 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 μ m (*left panels*) and 200 μ m (*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 μ m. *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* was measured per each image. Data are means $\pm$ SE for a total of five mice per group in three independent experiments. *D*, The absolute numbers of B cells (B220⁺), CD4⁺ T cells (CD4⁺), and CD8⁺ T cells (CD8⁺) in the spleen of WT or MT mice were determined by flow cytometry. Data are means $\pm$ SE for four mice per group and representative of three independent experiments. ** $p < 0.01$ (Student *t* test).

FIGURE 2.

Reduced expression of CCL19, CCL21, and IL-7 in the spleen of SIRP α MT mice. Expression of CCL19 (*Ccl19*), CCL21 (*Ccl21*), or CXCL13 (*Cxcl13*) mRNA (**A**) and IL-7 (*Il7*) mRNA (**B**) in the spleen of WT or SIRP α 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 $\pm$ SE for a total of 10 mice per group in three independent experiments. **C**, Frozen sections of the spleen from WT or SIRP α MT mice were double-stained with pAbs to CCL19 (*left panels*, red), CCL21 (*middle panels*, red), or CXCL13 (*right panels*, red) and an mAb to B220 (*left and middle panels*, green) or Thy1.2 (*right panels*, green). Scale bar, 200 μm . **D**, Frozen sections of the spleen from WT or SIRP α MT mice were stained with mAbs to gp38 (red) and B220 (green). Scale bar, 200 μm (*left panels*). The area for gp38-positive region was measured per each image. Data are means $\pm$ SE for a total of four (WT) or three (MT) mice per group (*right panel*). * $p < 0.05$, ** $p < 0.01$ (Student *t* test).

FIGURE 3.

Importance of CD47 for development of the splenic T cell zone. *A*, Paraffin sections of the spleen from WT or CD47-deficient (KO) mice were stained with H&E. Scale bar, 200 μm. *B*, Frozen sections of the spleen from WT or CD47 KO mice were stained with mAbs to B220 (green) and Thy1.2 (red). Scale bar, 500 μm (*left 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 for a total of six mice per group (*right panel*). *C*, The absolute numbers of B cells (B220⁺), CD4⁺ T cells (CD4⁺), and CD8⁺ T cells (CD8⁺) in the spleen of WT or CD47 KO mice were determined by flow cytometry. Data are means ± SE from three mice per group and representative of three independent experiments. Expression of CCL19 (*Ccl19*), CCL21 (*Ccl21*), or CXCL13 (*Cxcl13*) mRNA (*D*) or IL-7 (*Il7*) mRNA (*E*) in the spleen of WT or CD47 KO 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 five mice per group in three independent experiments. **p* < 0.05, ***p* < 0.01 (Student *t* test).

FIGURE 4.

Importance of hematopoietic SIRP α for development of the splenic T cell zone. **A**, WT or SIRP α MT mice were lethally irradiated and then reconstituted with 5×10^5 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 $\pm$ SE of eight to nine mice per group in two independent experiments (*lower panels*). Expression of CCL19 (*Ccl19*), CCL21 (*Ccl21*) or CXCL13 (*Cxcl13*) 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 $\pm$ 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).

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FIGURE 5.

Reduced expression of LT α , LT β , or LT β R and impaired LT signaling in the spleen of SIRP α MT mice. *A*, Expression of mRNA for LT α (*Lta*), LT β (*Ltb*), LIGHT (*Tnfsf14*), or LT β R (*Ltbr*) in the spleen of WT or SIRP α 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 $\pm$ SE for a total of six mice per group in three independent experiments. *B*, Total RNA was extracted from T cells or B cells isolated from the spleen of WT or SIRP α MT mice, and the expression of LT α or LT β mRNA was analyzed 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 T cells or B cells. Data are means $\pm$ SE for a total three mice per group in three independent experiments. *C*, WT or SIRP α MT mice were injected i.p. with the agonistic mAb to LT β R or control IgG. Twenty-four hours after injection, spleens were harvested, and the expression of CCL21 (*Ccl21*) or NF κ B p100 (*Nfkb2*) mRNA was analyzed 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 treated with control IgG. Data are means $\pm$ SE for three mice per group and are representative from two independent experiments. * p < 0.05, ** p < 0.01 (Student *t* test).