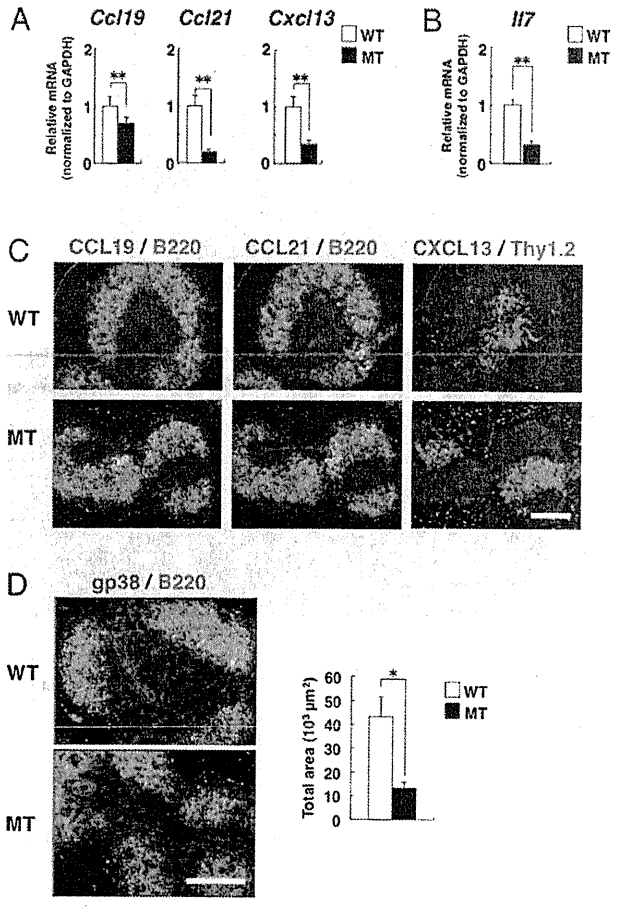

FIGURE 2. Reduced expression of CCL19, CCL21, and IL-7 in the spleen of SIRPα MT mice. Expression of CCL19 (*Ccl19*), CCL21 (*Ccl21*), or CXCL13 (*Cxcl13*) mRNA (*A*) and IL-7 (*Il7*) mRNA (*B*) in the spleen of WT or SIRPα MT mice was evaluated by quantitative PCR. The level of expression of each mRNA 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. *C*, Frozen sections of the spleen from WT or SIRPα 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 SIRPα 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 ± SE for a total of four (WT) or three (MT) mice per group (*right panel*). $*p < 0.05$, $**p < 0.01$ (Student *t* test).

significantly suppressed, consistent with a stromal cell phenotype in SIRPα MT mice (Fig. 2*D*). Expression levels of mRNA for CCL19, CCL21, CXCL13, and IL-7 in the pLNs of SIRPα MT mice did not differ between WT and SIRPα 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 phenocopy of the SIRPα MT mice (Fig. 3*A*). Consistently, CD47 KO mice showed a loss in the area of the T cell zone in the spleen (Fig. 3*B*) and decreased numbers of $CD4^+$ T cells (Fig. 3*C*) and the expression of CCL19, CCL21, and CXCL13 and IL-7 mRNA (Fig. 3*D*, 3*E*).

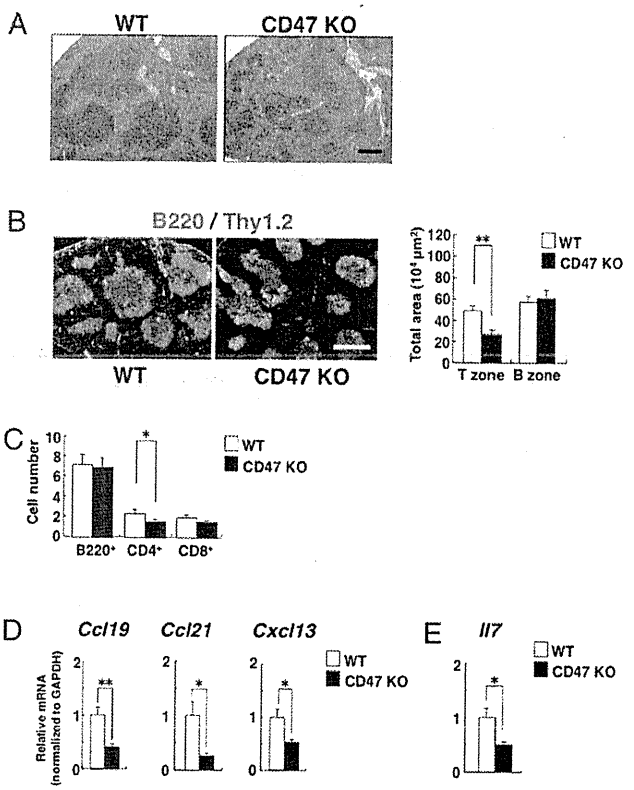

FIGURE 3. Importance of CD47 for development of the splenic T cell zone. *A*, Paraffin sections of the spleen from WT or CD47-deficient (KO) mice were stained with H&E. Scale bar, 200 μm. *B*, Frozen sections of the spleen from WT or CD47 KO mice were stained with mAbs to B220 (green) and Thy1.2 (red). 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).

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. 4*A*). 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. 4*A*). By contrast, the area of the T cell zone, as well as that for B cell follicles, in the spleen was similar

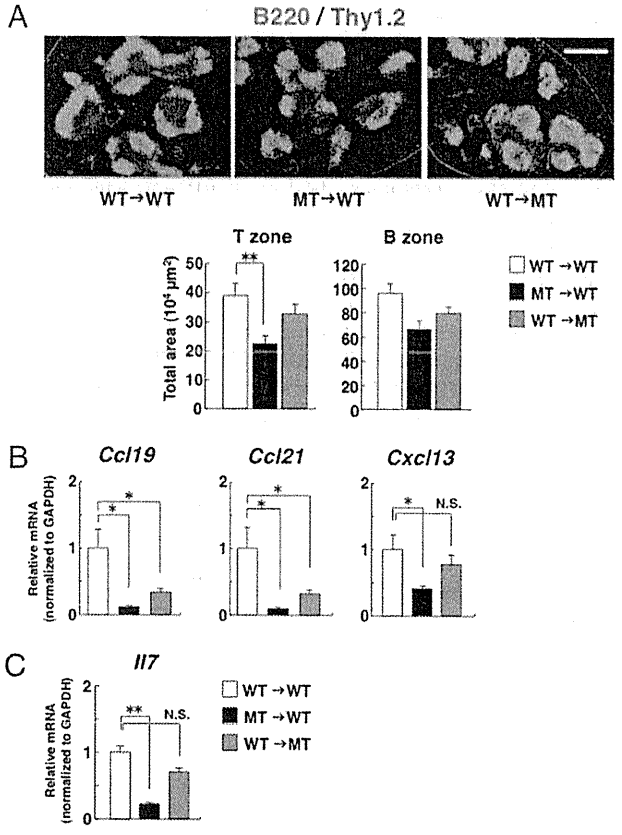

FIGURE 4. Importance of hematopoietic SIRPα for development of the splenic T cell zone. *A*, WT or SIRPα MT mice were lethally irradiated and then reconstituted with 5×10^5 BM cells from WT or MT mice for generating WT→WT, WT→MT, or MT→WT chimeras. Eight weeks after transplantation, spleens were harvested, 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 ± 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 ± 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. 4*A*). 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. 1*A*). 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. 4*B*). In addition, the mRNA expression levels of CCL19 and CCL21, but not that of CXCL13, were also decreased in WT→MT chimeras (Fig. 4*B*). Furthermore, IL-7 mRNA expression was also decreased in the spleen of MT→WT chimeras, but not in WT→MT chimeras (Fig. 4*C*).

Reduced expression of LTα, LTβ, or LTβR and impaired LT signaling in the spleen of SIRPα 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 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. 5*A*). 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. 5*B*).

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

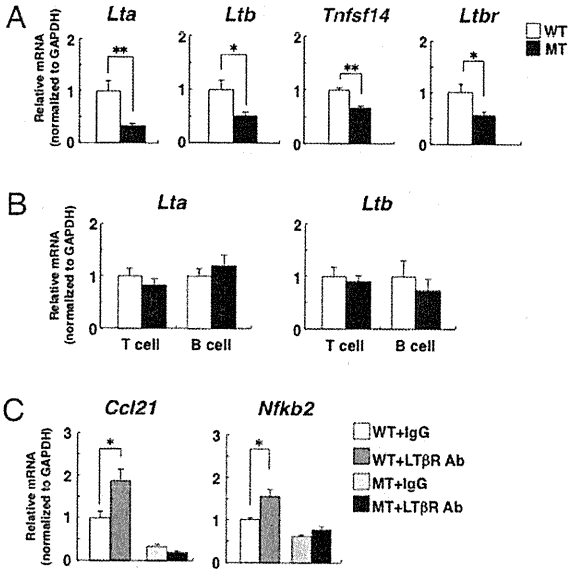

FIGURE 5. Reduced expression of LTα, LTβ, or LTβR and impaired LT signaling in the spleen of SIRPα MT mice. *A*, Expression of mRNA for LTα (*Lta*), LTβ (*Ltb*), LIGHT (*Tnfsf14*), or LTβR (*Ltbr*) in the spleen of WT of SIRPα MT mice was evaluated by quantitative PCR. The level of expression of each 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 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 SIRPα MT mice, and the expression of LTα or LTβ 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 ± SE for a total three mice per group in three independent experiments. *C*, WT or SIRPα MT mice were injected i.p. with the agonistic mAb to LTβR or control IgG. Twenty-four hours after injection, spleens were harvested, and the expression of CCL21 (*Ccl21*) or NFκb2 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 ± SE for three mice per group and are representative from two independent experiments. $*p < 0.05$, $**p < 0.01$ (Student *t* test).

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. 5*C*). By contrast, such increase of CCL21 mRNA expression was not apparent in the spleen of SIRPα MT mice (Fig. 5*C*). 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. 5*C*).

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 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 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 of CCL19 or CCL21 from FRCs. Indeed, the spleens of adult $LT\alpha^{-/-}$, $LT\beta^{-/-}$, and $LT\beta R^{-/-}$ 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 LTαβ 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 independent of 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. 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 SIRPα MT mice. By contrast, the number of $CD11c^{high}$ 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).

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 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.

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Essential roles of SIRPα in homeostatic regulation of skin dendritic cells

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ABSTRACT

Signal regulatory protein α (SIRPα) is an immunoglobulin superfamily protein that is predominantly expressed in dendritic cells (DCs). Its cytoplasmic region binds SHP-1 or SHP-2 protein tyrosine phosphatases, while its extracellular region interacts with CD47, another immunoglobulin superfamily protein, constituting cell-cell signaling. SIRPα was previously shown to be important for development of contact hypersensitivity, likely as a result of its positive regulation of the priming by DCs of $CD4^+$ T cells. However, the mechanism by which SIRPα regulates DC functions remains unknown. Here we found that the number of $I\text{-}A^+$ cells, which represent migratory DCs such as Langerhans cells (LCs) or dermal DCs from the skin, in the peripheral lymph nodes (LNs) was markedly decreased in mice expressing a mutant form of SIRPα that lacks the cytoplasmic region compared with that of wild-type (WT) mice. In addition, an increase of fluorescein isothiocyanate (FITC)-bearing $I\text{-}A^+$ cells in the draining lymph nodes (LNs) after skin-painting with FITC was markedly blunted in SIRPα mutant mice. However, migratory ability, as well as expression of CCR7, of bone marrow-derived DCs prepared from SIRPα mutant mice were not impaired. By contrast, the number of $I\text{-}A^+$ LCs in the epidermis of SIRPα mutant mice was markedly decreased compared with that of WT mice. In addition, the mRNA expression of transforming growth factor-β receptor II in LCs of SIRPα mutant mice was markedly decreased compared with that of WT mice. These results suggest that SIRPα is important for homeostasis of LCs in the skin, as well as of migratory DCs in the LNs, but unlikely for migration of these cells from the skin to draining LNs.

1. Introduction

Contact hypersensitivity (CHS) is an inflammatory immune response of skin that is induced by the local application of haptens. Epidermal dendritic cells (DCs) as well as dermal DCs in the skin are professional antigen presenting cells that play a central role in the induction of CHS [1–3]. Epidermal DC are also known as Langerhans cells (LCs) that are $CD11c^+$ $Langerin^+$ MHC class II^+ DCs, whereas dermal DCs belong to a broad subset of interstitial MHC class II^+ DCs [1,2]. When these cells, which reside in the skin, encounter exogenous antigens, they engulf and process them for presentation of antigen-derived peptides in complexes with MHC molecules on the cell surface. The LCs or dermal DCs subsequently migrate to draining lymph nodes (LNs) and make contact with naive T cells. During such migration, these DCs mature and express costimulatory molecules such as CD80, CD86, and CD40 in addition to MHC class II molecules on their surface [1]. In addition, upregulation by DCs of CCR7 is essential for their migration to LNs in response to CCR7 ligands, such as CCL19 or CCL21 [4–7]. The mature DCs thus present MHC-peptide complexes to naive T cells together with costimulatory molecules that are essential for priming of the T cells by DCs [1,8]. Interleukin (IL)-17 production by $CD4^+$ T helper (Th) cells known as Th17 cells was recently shown to be essential for development of CHS, as well as for that of other autoimmune diseases

Abbreviations: CHS, contact hypersensitivity; DC, dendritic cell; LCs, Langerhans cells; LNs, lymph nodes; IL, Interleukin; Th, T helper; SIRPα, signal-regulatory protein α; Ig, immunoglobulin; DNFB, 2,4-dinitro-1-fluorobenzene; EAE, experimental autoimmune encephalomyelitis; WT, wild-type; FITC, fluorescein isothiocyanate; BM, bone marrow; BMDCs, BM-derived DCs; mAb, monoclonal antibody; PE, phycoerythrin; GM-CSF, granulocyte macrophage colony-stimulating factor; PI, propidium iodide; LPS, lipopolysaccharide; CFSE, 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester; CMTMR, 5-(and-6)-(((4-chloromethyl)-benzyol) amino) tetramethyl-rhodamine; OVA, ovalbumin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TGFβRII, transforming growth factor-β receptor II; M-CSFR, macrophage colony-stimulating factor receptor; cDC, conventional DCs.

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[1,9–11]. Indeed, IL-17-deficient mice manifest reduced susceptibility to CHS [12]. However, the molecular mechanism by which LCs or dermal DCs prime naive $CD4^+$ T cells and induce their differentiation into Th17 cells during the development of CHS remains largely unknown.

Signal regulatory protein α (SIRPα) [13,14], also known as Src homology 2 domain-containing protein tyrosine phosphatase substrate-1 (SHPS-1) or BIT, is a transmembrane protein whose extracellular region comprises three immunoglobulin (Ig)-like domains and whose cytoplasmic region contains four tyrosine phosphorylation sites that mediate binding of the protein tyrosine phosphatases SHP-1 and SHP-2. Tyrosine phosphorylation of SIRPα is regulated by various growth factors and cytokines as well as by integrin-mediated cell adhesion to extracellular matrix protein. SIRPα thus functions as a docking protein to recruit and activate SHP-1 or SHP-2 at the cell membrane in response to extracellular stimuli, and these protein phosphatases are thought to be important for signaling downstream of SIRPα. The extracellular region of SIRPα interacts with its ligand, CD47, which is also a member of the Ig superfamily, with such interaction promoting the tyrosine phosphorylation of SIRPα [13–15].

Among hematopoietic cells, SIRPα is especially abundant in DCs, LCs, and macrophages, being barely detectable in T or B lymphocytes [13,16–19]. In contrast, CD47 is expressed in a variety of hematopoietic cells including red blood cells and T cells [13–15]. We have recently shown that mice expressing a mutant form of SIRPα that lacks most of the cytoplasmic region are resistant to 2,4-dinitro-1-fluorobenzene (DNFB)-induced CHS as well as to experimental autoimmune encephalomyelitis (EAE) or collagen-induced arthritis [20–22]. In addition, the production of IL-17 by T cells from SIRPα mutant mice exposed to the antigens responsible for the induction of these conditions was markedly impaired, suggesting that SIRPα expressed on the surface of DCs is essential for priming of naive T cells as well as for their Th17 differentiation during the development of CHS or EAE. However, the molecular mechanism by which SIRPα regulates DC functions for Th17 differentiation remains poorly understood.

It was previously suggested that SIRPα is important for development of CHS through regulation of DC migration from the epidermis to draining LNs in response to DNFB [16,20,23]. Indeed, interaction of SIRPα with its ligand CD47 is also thought to regulate the transmigration of neutrophils or monocytes as well as migration of melanoma cells [24–26]. We have now re-evaluated whether SIRPα is indeed important for regulation of DC migration. We here found that the number of migratory DCs in the peripheral LNs of SIRPα mutant mice was markedly decreased compared with that apparent with wild-type (WT) mice. Moreover, the number of fluorescein isothiocyanate (FITC)-labeled skin DCs in the draining LNs after hapten FITC painting was markedly reduced in SIRPα mutant mice. However, in vivo or in vitro migration of bone marrow (BM)-derived DCs (BMDCs), which were prepared from SIRPα mutant mice, did not differ from that apparent with BMDCs prepared from WT mice. By contrast, the absolute number of LCs in the epidermis of SIRPα mutant mice was markedly decreased, suggesting that SIRPα is important for homeostasis of LCs in the skin.

2. Materials and methods

2.1. Antibodies and reagents

A rat monoclonal antibody (mAb) to mouse SIRPα (p84; kindly provided by C. F. Lagenaur, University of Pittsburgh, PA, USA) was purified from culture supernatants of hybridoma cells. The mAbs were conjugated to sulfo-NHS-LC biotin [sulfosuccinimidyl-6-(biotinamido) hexanoate; Pierce]. 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). FITC-conjugated mAbs to CD11c (HL3), to CD4 (RM4-5), to I-A (AF6-120.1), or to CD45.2 (104); biotin-conjugated rat IgG to trinitrophenol (isotype control); and phycoerythrin (PE)-conjugated streptavidin were obtained from BD Biosciences. FITC-conjugated rat IgG to trinitrophenol (isotype control) and biotin-conjugated mAbs to CCR7 (4B12) or I-A (M5/114.15.2) were from eBioscience. CD4 (L3T4) microbeads were from Miltenyi Biotech. Recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF) and recombinant murine MIP-3 (CCL19) were from Peprotech. Propidium Iodide (PI), lipopolysaccharide (LPS) and FITC isomer I were obtained from Sigma-Aldrich. 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) and 5-(and-6)-(((4-chloromethyl)-benzyol) amino) tetramethylrhodamine (CMTMR) were purchased from Invitrogen. Ovalbumin (OVA) was obtained from Calbiochem. RPMI 1640 medium (Sigma-Aldrich) was supplemented with 10% heat-inactivated fetal bovine serum, 50 μM 2-mercaptoethanol, 10 mM HEPES-NaOH (pH 7.4), penicillin (100 U/ml), streptomycin (100 μg/ml), and 1 mM sodium pyruvate to yield complete medium.

2.2. Mice

Mice expressing a mutant version of SIRPα that lacks most of the cytoplasmic region were described previously [18,21,27] and were backcrossed to the C57BL/6 background for five generations. OT-II TCR transgenic mice ($H\text{-}2^b$), which had been originally generated by Barnden et al. [28], were kindly provided from Dr. T. Hirano (Riken, Yokohama, Japan). Sex- and age-matched mice at 6 to 10 weeks of age were studied. Mice were bred and maintained at the Institute of Experimental Animal Research of Gunma University under specific pathogen-free conditions and were handled in accordance with the animal care guidelines of Gunma University.

2.3. Cell preparation and flow cytometry

Cell suspensions were prepared from draining LNs as described previously [21]. In brief, LNs 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). The remaining cells were washed twice with phosphate-buffered saline (PBS). For flow cytometric analysis, cells were first incubated with a mAb to mouse CD16/32 to prevent nonspecific binding of labeled mAbs to Fcγ receptors and thereafter labeled with specific mAbs. Cells were then analyzed by flow cytometry with the use of a FACSCalibur (BD Biosciences), and all data were analyzed with FlowJo software (Tree Star).

2.4. FITC sensitization

Sensitization of the skin with FITC to determine the migration of cutaneous DCs to the draining LNs was performed as described previously [16,29]. Briefly, FITC was dissolved in a 50/50 (v/v) acetone: dibutylphthalate mixture just before application. Thereafter, mice were painted on the shaved thorax and abdomen with 200 μl of 1% FITC solutions or vehicle alone. Twenty-four h after painting, the inguinal and axillary LNs were isolated and digested with collagenase as described above. Cells were washed, incubated with a biotin-conjugated mAb to mouse I-A, followed by staining with PE-conjugated streptavidin. The cells were washed again, stained with PI, and then analyzed by flow cytometry.

2.5. Generation of BMDCs

BMDCs were prepared by culture of BM cells with GM-CSF as described previously [21]. In brief, BM cells were isolated from the femur and tibia with the use of a syringe fitted with a 23-gauge needle. Fibrous material was removed by filtration through a 70-μm cell strainer, and red blood cells in the filtrate were lysed with Gey's solution. The remaining cells were washed twice with PBS. BM cells (1×10^6/ml) in RPMI 1640 complete medium supplemented with GM-CSF (10 ng/ml) were seeded in a 24-well culture plate, and the culture medium was changed every 2 days in order to remove granulocytes. After culture for 7 days, loosely adherent and clustered cells were collected as immature BMDCs. Immature BMDCs were further incubated with 1 μg/ml of LPS for 24 h to obtain mature BMDCs. To determine the surface expression of CCR7 on BMDCs, the cells were first incubated with a biotin-conjugated mAb to mouse CCR7 or isotype matched control, and were then incubated with an FITC-conjugated mAb to mouse I-A and PE-conjugated streptavidin. Cells were washed again, incubated with PI, and then analyzed by flow cytometry.

2.6. In vitro chemotaxis assay

Chemotaxis in response to CCL19, a CCR7 ligand, was determined as described previously with minor modifications [16,29]. In brief, mature BMDCs (3×10^5) prepared from WT or SIRPα mutant mice were suspended in 100 μl of RPMI 1640 containing 0.1% bovine serum albumin (BSA) and were then placed on the upper compartment of 5-μm pore size Transwell® plates (Corning). CCL19 (at concentration of 250 ng/ml) in 600 μl of RPMI containing 0.1% BSA was added to the lower chamber. The cells were then incubated for 2 h at 37 °C under 5% CO_2, after which cells that transmigrated to the bottom compartment were collected and the cell number was counted by flow cytometry.

2.7. In vivo migration assay

To examine the migratory ability of DCs in vivo, matured BMDCs that were prepared from WT or SIRPα mutant mice were labeled with 10 μg/ml CFSE for 10 min or 20 μg/ml CMTMR for 30 min, respectively, and *vice versa* at 37 °C in RPMI 1640 supplement with 0.5% BSA. The cells were then washed twice, mixed at a ratio of 1:1 (2×10^6 cells in 40 μl RPMI 1640), after which the labeled cells were injected subcutaneously into the footpad of WT mice. Twenty-four h after injection, the LN cells were prepared from the popliteal LNs and then subjected to flow cytometrical analysis.

2.8. Assay for proliferation of $CD4^+$ T cells transferred from OT-II mice

To prepare splenic $CD4^+$ T cells from OT-II TCR transgenic mice [21,28], the spleen was gently ground with autoclaved glass slides in PBS, and the released cells were exposed to Gey's solution, washed twice with PBS, and filtered through nylon wool. Cells in the filtrate were then subjected to purification with the use of anti-mouse CD4 (L3T4) microbeads and magnetic-activated cell sorting (Miltenyi Biotech). The purity of the isolated $CD4^+$ T cell was > 95% as determined by flow cytometry. The cells were then labeled with 10 μg/ml CFSE in RPMI 1640 supplement with 0.5% BSA for 10 min at 37 °C, washed, and injected intravenously into WT or SIRPα mutant mice (2×10^6 cells in 500 μl of RPMI 1640). Twenty-four h after injection, these mice were immunized subcutaneously in the footpad with 10 μg of OVA dissolved in 40 μl of PBS. Forty-eight h after the immunization, popliteal LNs were isolated and treated with collagenase as described above. The cells were washed, incubated with PI, and then analyzed by flow cytometry. Proliferation of transferred OT-II $CD4^+$ T cells that was measured by CSFE dilution was determined by the percentage of undivided cells (see Fig. 5).

2.9. Preparation of epidermal sheets and immunofluorescence analysis

Epidermal sheets were prepared as previously described [16]. Briefly, ears were split into dorsal and ventral halves, and the dorsal ear halves were incubated with 2 M NaBr for 2 h at 37 °C. The epidermis was then separated from the dermis using forceps and was washed in PBS. Epidermal sheets were then fixed in acetone for 3–5 min at −20 °C. After fixation, the sheets were washed in PBS and then incubated with a biotin-conjugated mAb to mouse I-A at room temperature for 30 min, followed by incubation with FITC-conjugated streptavidin (diluted at 1/100 in 5% bovine serum/PBS) for 30 min, and washed with PBS. Images for I-A positive cells were captured with a fluorescence microscope and subjected to the analysis by ImageJ software (National Institutes of Health)

2.10. Preparation of epidermal and dermal cells and analysis by flow cytometry

Epidermal and dermal cells were prepared by modification of a method for preparation of epidermal cells as described previously [16]. In brief, dorsal and ventral ear halves were incubated in 1% trypsin (Difco) in PBS for 60 min at 37 °C. The epidermis and dermis were gently separated and were then shaken vigorously in PBS. After the solution became cloudy, a single-cell suspension was prepared by filtration through a 70-μm cell strainer and washed twice in PBS. The cell suspensions were then incubated with a biotin-conjugated mAb to I-A and further incubated with an FITC-conjugated mAb to mouse CD45.2, PE-conjugated streptavidin and PI. To examine the expression of SIRPα, the cells were incubated with a biotin-conjugated mAb to SIRPα or isotype control, and then stained with an FITC-conjugated mAb to mouse I-A, followed by incubation with PE-streptavidin and PI. The cell suspensions were then washed and analyzed by flow cytometry.

2.11. Preparation of cDNA from LCs and quantitative real-time PCR

LCs were prepared and stained as described above, and were then sorted as $CD45^+$ I-A^+ PI^- cells by the use of the FACSAriaII instrument (BD Biosciences). Total RNA was extracted from the purified LCs using RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. The first strand cDNA was synthesized from total RNA using QuantiTect™ Reverse Transcription kit (QIAGEN) according to the manufacture's instructions. cDNA fragments of interest were amplified using 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 and were then normalized with glyceraldehydes-3-phosphate dehydrogenase (GAPDH) levels for each sample. Primer sequences for quantitative real-time PCR were as follows: *Tgfbr2*, forward: 5′-ATCTGGAAAACGTGGAGTCG-3′, reverse: 5′-TCCTTCACTTCTCCCACAGC-3′, *Csf1r* forward; 5′-GCGCAAGCTTGAGTTTATCACCCA-3′, reverse: 5′-ATATCGCAGGG-TGAGCTCAAAGGT-3′.

2.12. Statistical analysis

Data are presented as means ± SE and were analyzed by Student's *t* test. A *P* value of <0.05 was considered statistically significant.

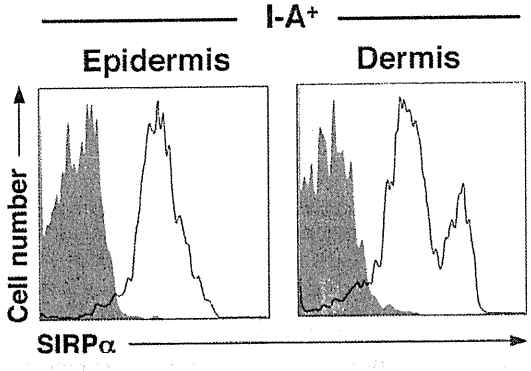

Fig. 1. Expression of SIRPα in LCs or dermal DCs. Epidermal or dermal cells prepared from wild-type mice were incubated with a biotin-conjugated mAb to SIRPα or isotype control, and then stained with an FITC-conjugated mAb to mouse I-A, followed by staining with PE-conjugated streptavidin and PI. The expression of SIRPα (open traces) or staining with the isotype control (filled traces) on I-A$^+$ PI$^-$ cells in the epidermis (left panel) or in the dermis (right panel) was shown in the histograms. Data are representative from three independent experiments.

3. Results

3.1. A marked reduction of I-A$^+$ cells, as well as that of FITC-bearing I-A$^+$ cells, in the draining LNs of SIRPα mutant mice subjected to cutaneous sensitization with FITC

Local application of FITC, as a hapten, in the skin is known to cause an inflammatory immune response as a model for CHS. It induces robust migration of skin DCs, such as LCs or dermal DCs, to draining LNs [16,29,30]. We previously showed that SIRPα is expressed on I-A$^+$ epidermal LCs [16], and we have confirmed this observation in the present study (Fig. 1). I-A$^+$ dermal DCs also expressed SIRPα, they are heterogenous with regard to the expression level of SIRPα, however (Fig. 1). To evaluate the importance of SIRPα for regulation of DC migration, we first examined the impact of cutaneous sensitization with FITC in WT and SIRPα mutant mice. The SIRPα mutant mice express a mutant version of SIRPα that lacks most of the cytoplasmic region [18,21]. This mutant protein does not undergo tyrosine phosphorylation or form a complex with SHP-1 or SHP-2. 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 mutant mice [18,21,27]. Furthermore, the cellular abundance of the mutant protein is markedly reduced compared with that of the full-length protein in WT cells [18,21]. The total cell number in the axillary and inguinal LNs of SIRPα mutant mice was not markedly different from that of WT mice (Fig. 2A). FITC painting at the thorax and abdomen increased the total cell number in the draining LNs of WT mice, whereas such increase was markedly diminished in the LNs of SIRPα mutant mice (Fig. 2A). In addition, the number of I-A$^+$ cells, which represent migratory DCs such as LCs or dermal DCs from the skin [2], was markedly decreased in the draining LNs of SIRPα mutant mice with or without FITC painting, compared with that of WT animals (Fig. 2B). Moreover, the number of FITC-bearing I-A$^+$ cells in the LNs of SIRPα mutant mice was markedly decreased compared with that of WT mice (Fig. 2C). These data suggest that SIRPα is important for the steady-state homeosta-

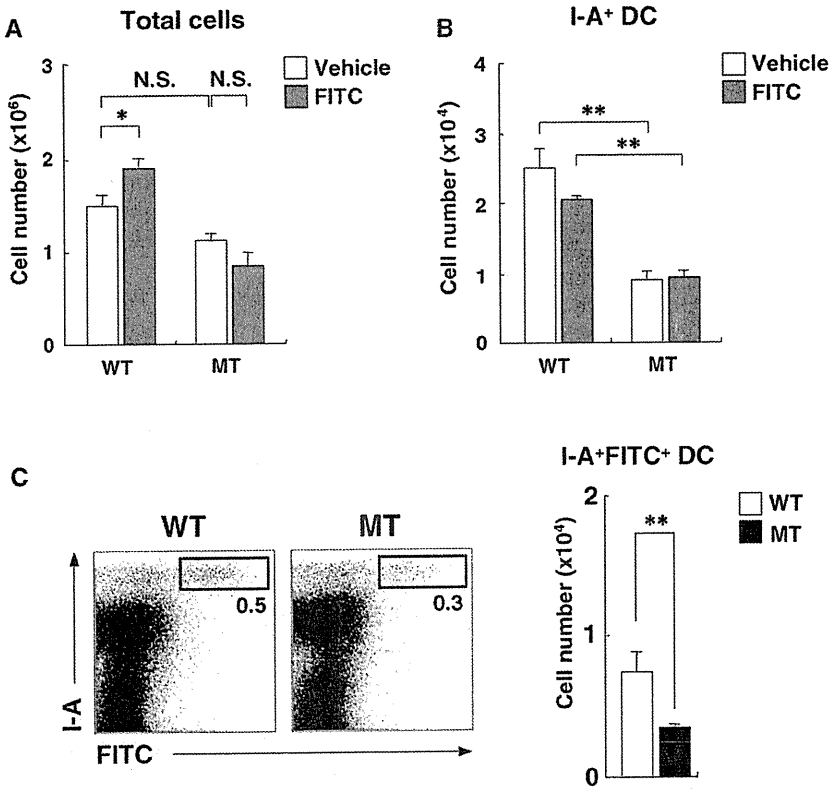

Fig. 2. A marked reduction of I-A$^+$ cells, as well as that of FITC-bearing I-A$^+$ cells, in the draining LNs of SIRPα mutant mice subjected to cutaneous sensitization with FITC. Wild-type (WT) or SIRPα mutant (MT) mice were painted on the shaved thorax and abdomen with 200 μl of 1% of FITC solutions or vehicle alone. Twenty-four h after painting, cells were prepared from inguinal and axillary LNs. (A) The total number of LN cells was determined by the use of a Burker–Turk counting chamber. Data are means ± SE of values from three mice per group and are from three independent experiments. N.S., not significant. *$P < 0.05$ (Student's t test). (B and C) Cells prepared as in (A) were incubated with a biotin-conjugated mAb to mouse I-A. The cells were stained with PE-conjugated streptavidin and PI, and then analyzed by three-color flow cytometry. The absolute numbers of I-A$^+$ DCs from WT or SIRPα mutant mice were then determined (B). The relative numbers of I-A$^+$ FITC$^+$ cells are also expressed as a percentage of all viable cells on each plot (C, left panels). The absolute numbers of I-A$^+$ FITC$^+$ cells in the draining LNs isolated from WT or SIRPα mutant mice were also determined (C, right panel). Data are means ± SE of values from three mice per group and are from three independent experiments. **$P < 0.01$ (Student's t test).

sis of I-A$^+$ migratory DCs, as well as an increase by FITC painting of these cells, in the draining LNs.

3.2. No impairment of migration of BMDCs prepared from SIRPα mutant mice

The reduction of FITC-bearing I-A$^+$ cells in the draining LNs of SIRPα mutant mice with FITC painting suggests that migration of I-A$^+$ cells from the skin to the draining LNs is likely impaired in these mutant mice. We previously showed that SIRPα is abundantly expressed in BMDCs [20]. We thus next examined the migration of BMDCs, which had been subcutaneously injected into the footpads, to the draining LNs. BMDCs were prepared from the BM of either WT or SIRPα mutant mice and activated with LPS, after which the BMDCs were labeled with CFSE (for WT) or CMTMR (for SIRPα mutant), respectively, and *vice versa* before subcutaneous injection to WT mice at 1:1 ratio (see the upper panels of Fig. 3). Twenty-four h after injection, the LN cells were prepared from the popliteal LNs and then subjected to flow cytometrical analysis. However, the frequency of migrated BMDCs that were prepared from SIRPα mutant mice in the draining LNs was equivalent to that apparent with WT BMDCs (Fig. 3).

Upregulation by activated DCs of CCR7 expression is thought to be important for migration of these cells from the skin to the draining LNs [6,7]. Treatment of BMDCs with LPS for 24 h markedly increased the surface expression of CCR7 (Fig. 4A). However, the expression level of CCR7 on the LPS-activated (matured) BMDCs of SIRPα mutant mice was equivalent to that of WT BMDCs (Fig. 4A). By the use of transwell chambers, we also determined the migratory response of BMDCs to CCL19, a ligand for CCR7 [6,7]. However, the migration of BMDCs of SIRPα mutant mice toward CCL19 did not differ from that of WT BMDCs (Fig. 4B). These results thus suggest that SIRPα is unlikely important for promotion of DC migration.

3.3. No impairment of proliferation of OT-II CD4$^+$ T cells transferred into SIRPα mutant mice

To further clarify the cause of the reduced number of FITC-bearing I-A$^+$ cells in the draining LNs of SIRPα mutant mice (Fig. 2), we next examined the antigen-specific proliferation of CD4$^+$ T cells from OT-II transgenic mice. Either WT or SIRPα mutant mice that had been transferred with CFSE-labeled CD4$^+$ T cells of OT-II mice were subcutaneously injected with OVA at footpads. Proliferation of transferred OT-II CD4$^+$ T cells, which was measured by CSFE dilu-

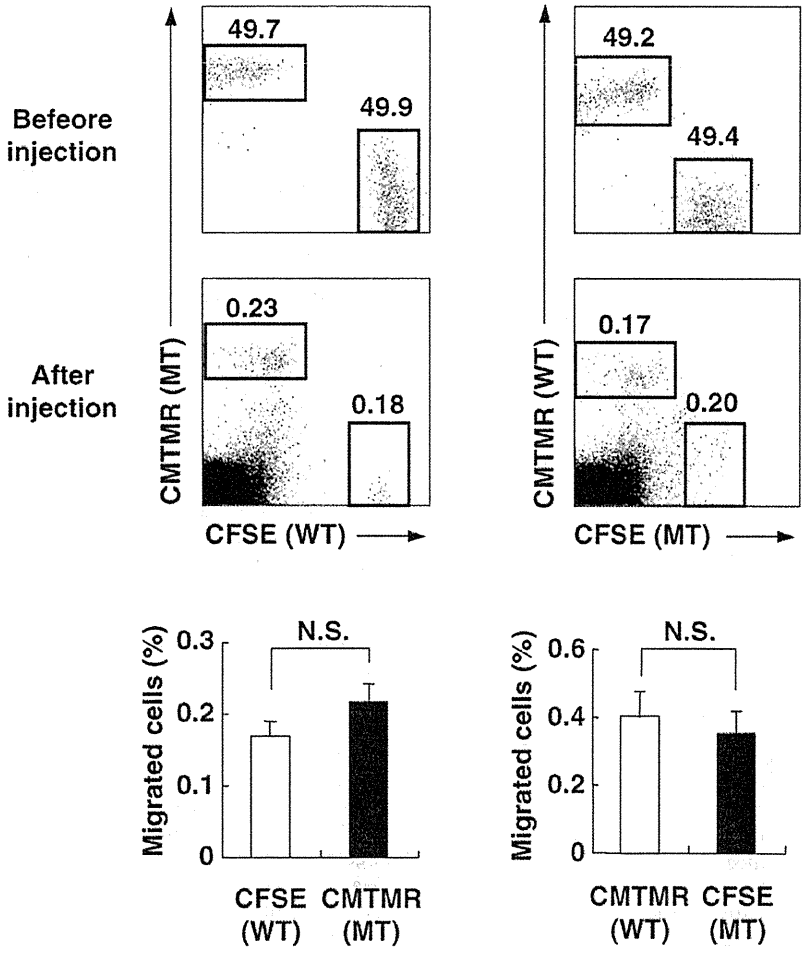

Fig. 3. No impairment of migration of BMDCs from SIRPα mutant mice. LPS-activated mature BMDCs derived from WT or SIRPα mutant mice were labeled with 10 μg/ml CFSE or 20 μg/ml CMTMR, respectively (left panels), and *vice versa* (right panels) in RPMI1640 containing 0.1% BSA. Thereafter, the cells were washed, mixed at 1:1 ratio, and then resuspended (2×10^6 cells in 40 μl) and injected into a hind footpad of WT mice. Twenty-four hours after injection, cells were prepared from the draining LNs and subjected to flow cytometric analysis for determination of the percentage of cells labeled with CFSE or CMTMR. The relative numbers of CFSE- or CMTMR-positive cells are expressed as a percentage of total mixed cells before injection in each plot (upper two panels) or as a percentage of total cells isolated from draining LNs (middle two panels). The percentages of CFSE- or CMTMR-positive migrated cells as gated on middle two panels among total cells were also determined (lower two panels). Data are means ± SE of values from three mice per group and representative from two independent experiments.

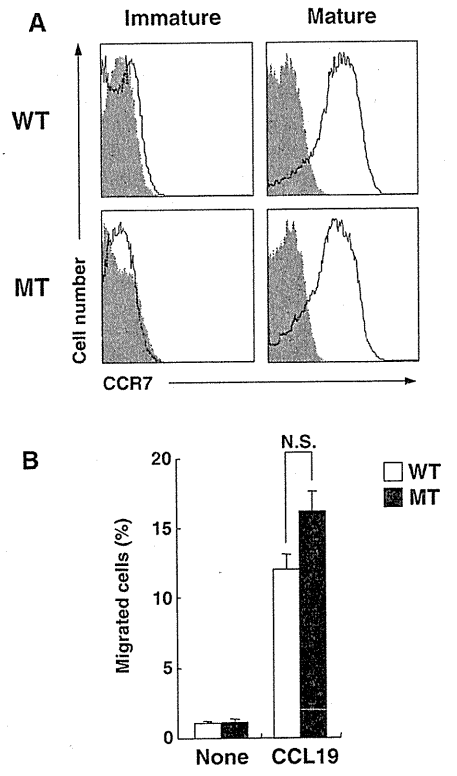

Fig. 4. No impairment of CCR7 expression, as well as chemotaxis to CCL19, of BMDCs from SIRPα mutant mice. (A) LPS-untreated (Immature) or -treated (Mature) BMDCs derived from WT or SIRPα mutant mice were incubated with a biotin-conjugated mAb to CCR7 or isotype control. The cells were also stained with an FITC-conjugated mAb to I-A, PE-conjugated streptavidin, and PI. The expression of CCR7 (open traces) or staining with the isotype control (filled traces) on I-A$^+$ PI$^-$ cells was analyzed by three-color flow cytometry. Data are representative of three independent experiments. (B) Mature BMDCs prepared from WT or SIRPα mutant mice were placed on the upper compartment of Transwell® chamber, and then RPMI containing 0.1% BSA with or without 250 ng/ml CCL19 was added to the lower chamber. Two h after incubation at 37 °C in 5% CO_2, cells that had migrated to the bottom compartment were collected and counted by flow cytometry. The relative number of migrated cells was determined as the percentage of cells collected from lower chamber among total subjected cells. Data are means ± SE of values from three mice per group and are from three separate experiments. N.S., not significant (Student's *t* test).

tion 48 h after OVA injection, in the popliteal LNs was not different between WT and SIRPα mutant mice (Fig. 5).

3.4. A reduction of I-A$^+$ cells in the epidermis of SIRPα mutant mice

We next examined the number of I-A$^+$ cells in the skin that represent LCs or dermal DCs [2] in SIRPα mutant mice. We found that the number of I-A$^+$ LCs in the epidermal sheets prepared from the ears of SIRPα mutant mice was markedly reduced compared to that of WT mice at 10 weeks of age (Fig. 6A). Moreover, we found that the number of I-A$^+$ LCs at 30 weeks of age was also decreased compared with that of age-matched WT mice (data not shown). In contrast, the frequency of I-A$^+$ dermal DCs, which include migrating LCs as well as Langerin-positive or -negative dermal DCs [1,2], in the dermis of the ears was not different between WT and SIRPα mutant mice (Fig. 6B).

To investigate the cause of the reduction of LCs in the epidermis of SIRPα mutant mice, we next evaluated the mRNA expression levels of transforming growth factor-β (TGF-β) receptor II (TGFβRII) and macrophage colony-stimulating factor receptor (M-CSFR; also known as CSF1R) in the LCs of SIRPα mutant mice. These receptors are able to bind TGF-β1 and M-CSF, respectively, and are thought to be crucial for development of LCs [31–33]. We found that the mRNA expression level of TGFβRII in the LCs of SIRPα mutant mice was markedly decreased compared with that of WT mice (Fig. 6C). In contrast, the mRNA expression level of CSF1R in the LCs of SIRPα mutant mice was similar to that of WT mice (Fig. 6C).

4. Discussion

We previously showed that SIRPα mutant mice are quite resistant to CHS that is induced by skin painting with hapten DNFB [20,23]. We here showed that skin painting with another hapten, FITC, promoted a marked increase of FITC-bearing I-A$^+$ DCs in the draining LNs in WT mice as described previously [20,23]. However, such increase was markedly blunted in SIRPα mutant mice. We also found that the number of I-A$^+$ DCs, which represent migratory DCs such as LCs or dermal DCs from the skin [2], was markedly decreased in the draining LNs of SIRPα mutant mice with or without FITC paining, compared with that of WT animals. These results suggest that SIRPα is likely important for promotion of migration of LCs or dermal DCs from the skin to the draining LNs in response to FITC. By contrast, SIRPα might be also important for homeostatic regulation of migratory DCs (LCs or dermal DCs) in the peripheral LNs.

Indeed, it was previously shown that injection of mAbs to SIRPα markedly prevented migratory response of I-A$^+$ LCs in the epidermis of WT mice in response to the painting with either DNFB or FITC [16]. Moreover, the reduction of LC population in the epidermis in response to DNFB painting was markedly attenuated in SIRPα mutant mice [16], suggesting that SIRPα is important for regulation of LC migration from the epidermis to draining LNs. However, we here showed that migration of subcutaneously injected BMDCs that were prepared from SIRPα mutant mice to the draining LNs was equivalent to that apparent with WT BMDCs. Moreover, LPS-induced expression of CCR7, as well as chemotactic migration of BMDCs toward CCL19, of SIRPα mutant mice were not impaired, compared with those apparent with WT BMDCs. Finally, we also found that proliferation of OT-II CD4$^+$ T cells, which had been transferred before injection of OVA into footpads, in the draining LNs was not different between WT and SIRPα mutant mice. Taken together, migratory ability, as well as priming capacity for OT-II CD4$^+$ T cells, of DCs of SIRPα mutant mice are unlikely impaired. Of interest is that migration of skin DCs to the draining LNs in response to FITC painting is markedly reduced in mice deficient of CD47, a ligand for SIRPα [29]. Moreover, in vivo migration of CD47-deficient BMDC or proliferation of transferred OT-II CD4$^+$ T cells in CD47-deficent mice was markedly impaired [29], although in vitro migration of CD47-deficient BMDCs toward CCL19 was equivalent to that of WT BMDCs. The authors thus indicated that CD47 expressed on DCs is important for lymphatic transendothelial DC migration [29]. Given the results of our study, CD47 on DCs might regulate DC migration in a manner independent of its interaction with SIRPα.

By contrast, we found that the number of I-A$^+$ LCs in the epidermis of SIRPα mutant mice was markedly reduced compared to that of WT mice, suggesting that SIRPα is important for homeostatic regulation of LCs in the epidermis under steady state conditions. The result also suggests that the reduction of I-A$^+$ migratory DCs, as well as of FITC-bearing I-A$^+$ DCs, in the draining LNs of SIRPα mutant mice is, at least in part, attributable to the reduction of LCs in the epidermis. Although the frequency of I-A$^+$ dermal DCs in the dermis was not different between WT and SIRPα mutant mice, dermal DCs consist of three distinct DC subsets, namely migrating LCs, Langerin$^+$ I-A$^+$ DCs and Langerin$^-$ I-A$^+$ DCs [1,2]. Thus, further analysis is required to examine whether one of such subsets of dermal DCs, such as migrating LCs, might be also reduced in SIRPα mutant

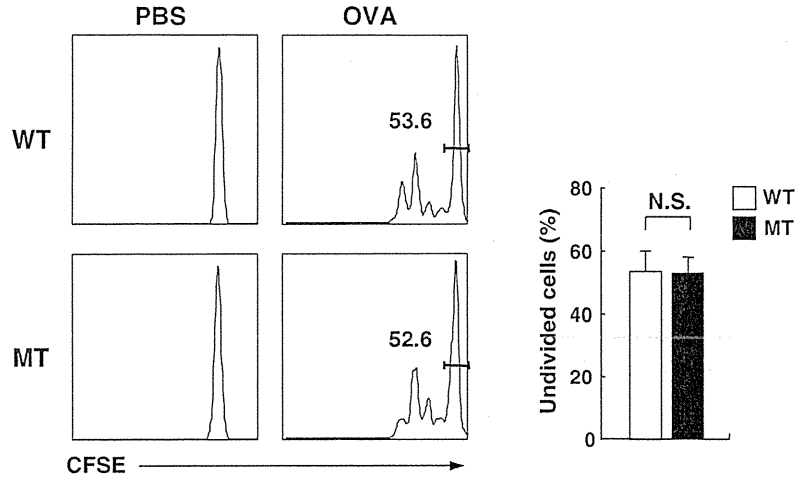

Fig. 5. No impairment of OVA-stimulated proliferation of OT-II CD4⁺ T cells adoptively transferred into SIRPα mutant mice. CD4⁺ T cells prepared from OT-II mice were labeled with 10 μg/ml CFSE, and the 2×10^6 of cells were injected intravenously into WT or SIRPα mutant mice. Twenty-four h after injection, these mice were immunized subcutaneously in the footpad with PBS or 10 μg of OVA. Forty-eight h after the immunization, LN cells were prepared from the draining LNs and subjected to flow cytometric analysis. Proliferation of transferred OT-II CD4⁺ T cells was determined by CFSE dilution. The relative numbers of $CFSE^{high}$ cells (undivided cells) are expressed as a percentage of CFSE-positive cells (left panels) and the percentage of undivided cells among all CFSE-positive cells were also determined (right panel). Data are means ± SE of values from three independent experiments. N.S., not significant (Student's *t* test).

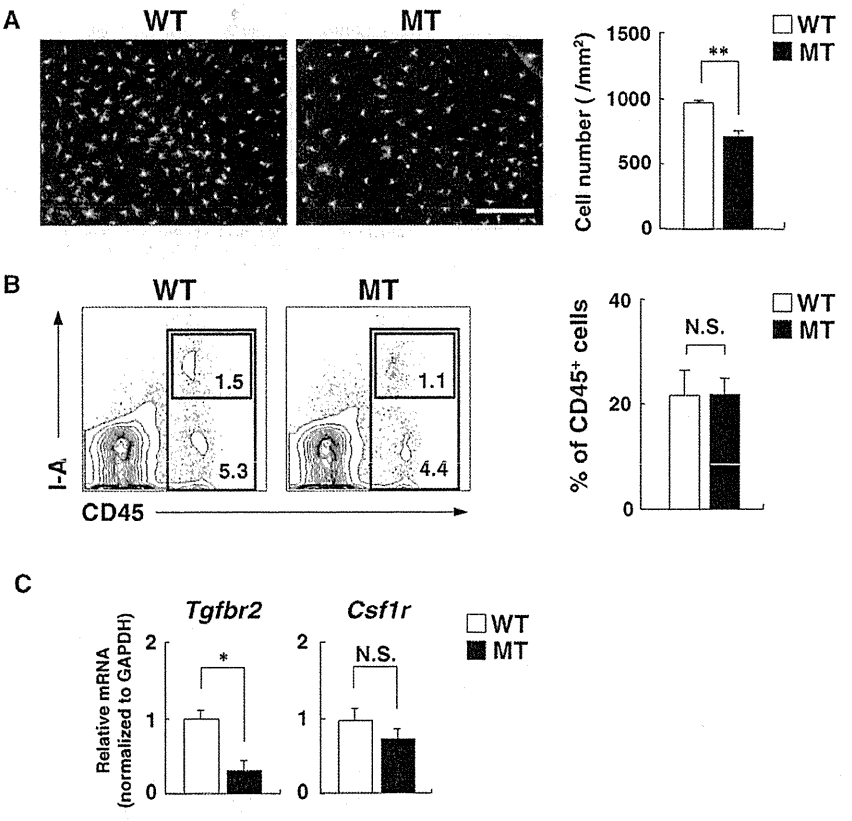

Fig. 6. A reduction of I-A⁺ cells in the epidermis of SIRPα mutant mice.
(A) Epidermal sheets prepared from WT or SIRPα mutant mice at 10 weeks of age were incubated with a biotin-conjugated mAb to mouse I-A, followed by staining with FITC-conjugated streptavidin. Images for I-A positive cells were captured with a fluorescence microscope. The number of I-A⁺ cells in the stained epidermal sheets was counted in five fields (for each epidermal sheet) and the average number per field was calculated. Data are means ± SE of values from four mice per group and are representative from five independent experiments. *$P < 0.05$ versus WT (Student's *t* test). Scale bar, 200 μm (B) Cells prepared from dermis of WT or SIRPα mutant mice were incubated with a biotin-conjugated mAb to I-A and then stained with an FITC-conjugated mAb to mouse CD45.2, PE-conjugated streptavidin and PI. The expression of CD45 and I-A cells among all viable cells was analyzed by three-color flow cytometry. The relative numbers of CD45⁺ I-A⁺ cells (inner box) among total CD45⁺ cells (outer box) are expressed as a percentage of viable cells. The percentage of CD45⁺ I-A⁺ cells among total CD45⁺ cells was also determined (right panel). Data are means ± SE of values from three mice per group and are representative from two independent experiments. N.S., not significant (Student's *t* test). (C) LCs were sorted from epidermal cells isolated from WT or SIRPα mutant mice (6 to10 mice per group). Total RNA was extracted and the mRNA expression of TGFβRII (*Tgfbr2*) or M-CSFR (*Csf1r*) was analyzed by real-time PCR. Data are means ± SE of values from three independent experiments. N.S., not significant. *$P < 0.05$ versus WT (Student's *t* test).

mice. Given that CD47, a ligand for SIRPα [13–15], is expressed in both hematopoietic and nonhematopoietic cells in the epidermal or dermal tissues (data not shown), *cis* interaction of CD47 with SIRPα within LCs or *trans* interaction of CD47 on other hematopoietic and nonhematopoetic cells with SIRPα on LCs might be important for the homeostatic regulation of LCs in the skin. Genetic analysis of LC development and homeostasis has revealed that several gene products regulate LC development, with some of them also controlling other subsets of $CD11c^+$ DCs in the secondary lymphoid tissues, however [2]. Development of LCs is markedly suppressed in mice deficient of TGF-β1 as well as its receptor TGFβRII [31,33] and M-CSFR [32]. Indeed, we found that the mRNA expression of TGFβRII was markedly reduced in LCs prepared from SIRPα mutant mice, suggesting that such reduction participates in the decrease of LCs in the epidermis of SIRPα mutant mice. Moreover, the result suggests that SIRPα is required for the expression of TGFβRII in LCs. LC development is also affected by genetic deficiency of transcription factors such as the helix-loop-helix transcription factor Id2 (inhibitor of DNA binding 2) [34] or interferon regulatory factor 8 [35], although the development of $CD8^+$ $CD11c^+$ conventional DCs (cDCs) in the secondary lymphoid tissues is also affected in these mutant animals. The present study have thus clarified that SIRPα is a new member of molecules that are important for homeostatic regulation of LCs in the skin.

We and others previously showed that the level of SIRPα expression differs among cDC subtypes, being greater in $CD8^-$ cDCs than in $CD8^+$ cDCs [17,19]. Furthermore, the number of $CD8^-$ cDCs, in particular that of $CD8^-$ $CD4^+$ cDCs, is selectively reduced in secondary lymphoid tissues of SIRPα mutant mice [19,36], suggesting that intrinsic SIRPα is required for homeostasis of $CD8^-$ $CD4^+$ cDC. Given that SIRPα is expressed in LCs, SIRPα might be also required intrinsically within LCs or LC precursors for normal accumulation of LCs in the skin. However, the molecular mechanism underlying regulation by SIRPα of LC homeostasis remains not elucidated yet and further investigation will be necessary to clarify such issue in the future.

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