SIRPα+ dendritic cells regulate homeostasis of fibroblastic reticular cells via TNF receptor ligands in the adult spleen

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Edited by Jason G. Cyster, University of California, San Francisco, CA, and approved October 16, 2017 (received for review June 24, 2017)

In secondary lymphoid organs, development and homeostasis of stromal cells such as podoplanin (Pdpn)-positive fibroblastic reticular cells (FRCs) are regulated by hematopoietic cells, but the cellular and molecular mechanisms of such regulation have remained unclear. Here we show that ablation of either signal regulatory protein α (SIRPα), an Ig superfamily protein, or its ligand CD47 in conventional dendritic cells (cDCs) markedly reduced the number of CD4+ FRCs as well as that of Pdpn+ FRCs and T cells in the adult mouse spleen. Such ablation also impaired the survival of FRCs as well as the production by CD4+ cDCs of tumor necrosis factor receptor (TNFR) ligands, including TNF-α, which was shown to promote the proliferation and survival of Pdpn+ FRCs. CD4+ cDCs thus regulate the steady-state homeostasis of FRCs in the adult spleen via the production of TNFR ligands, with the CD47–SIRPα interaction in cDCs likely being indispensable for such regulation.

In secondary lymphoid organs (SLOs) such as lymph nodes (LNs) and the spleen serve as sites for the interaction of a variety of hematopoietic cells in mammals. Dendritic cells (DCs) thus migrate from peripheral tissues and present antigens to T cells in SLOs, and SLOs are essential for the interaction of T cells with B cells. Such important functions of SLOs are supported by their stromal cell (SC) components (1–3). SCs contribute to the structural organization of SLOs by producing chemokines, which regulate the positioning and segregation of incoming hematopoietic cells. SCs in the white pulp of the spleen or paracortex of LNs are termed “fibroblastic reticular cells” (FRCs) and produce the chemokines CCL19 and CCL21, both of which attract naive T cells and activated DCs expressing the chemokine receptor CCR7 (4). In addition, FRCs produce IL-7, which is thought to support the survival of T cells and DCs (5). Moreover, FRCs produce extracellular matrix proteins such as collagens and laminins, and they form a tubular system, designated a “conduit system,” by which small molecules and fluid are transported from the marginal zone to the T cell area (6, 7). In contrast, follicular dendritic cells (FDCs), another subset of SCs, produce CXCL13 to attract B cells (1, 2).

Generation of SLO SCs is thought to require both hematopoietic cells and mesenchymal cells (2, 8). During fetal development of SLOs, mesenchymal precursors interact with lymphoid tissue-inducer (LTI) cells, which are derived from lymphoid precursors and belong to the family of innate lymphoid cells (9). The interaction of lymphotixin α1β2 (LTα1β2), a membrane-anchored heterotrimeric protein expressed on the surface of LTI cells, with lymphotixin β-receptor (LTβR) on mesenchymal precursors induces differentiation of the latter cells into stromal organizer cells (or lymphoid tissue organizer cells), which in turn are thought to give rise to various types of SCs through further interaction with hematopoietic cells. Differentiation of mesenchymal cells toward FDCs in SLOs is thought to require the presence of B cells, which express TNF-α as well as LTα1β2 (2). In addition, differentiation of FRCs requires LTα1β2—in particular, that expressed on B cells—in the spleen, but not in LNs (10). Systemic ablation of TNF-α was also shown to reduce the number of FRCs in the spleen (11, 12). However, which types of hematopoietic cells deliver specific signals important for the induction and homeostasis of FRCs in SLOs, especially under the steady-state condition in adults, has remained poorly understood.

Signal regulatory protein α (SIRPα) is an Ig superfamily protein that is highly expressed in CD11c+ conventional DCs (cDCs)—in particular, CD4+CD8α− cDCs (CD4+ cDCs)—as well as in macrophages (13, 14). SIRPα comprises three Ig-like domains in its extracellular region and an immunoreceptor tyrosine-based inhibition motif (ITIM) that binds the protein tyrosine phosphatases Shp1 and Shp2 in its intracellular region (15, 16). The extracellular region of SIRPα binds the ligand CD47, another membrane-bound protein of the Ig superfamily. CD47, which is highly expressed in CD11c+ conventional DCs (cDCs), mediates interactions with other cell types via its ligands, SIRPα and SIRPβ, a receptor of the immunoglobulin superfamily (17, 18). SIRPα is expressed on hematopoietic cells, including myeloid cells (19), B cells (20), and T cells (21, 22). SIRPα also mediates interactions with stromal cells such as podoplanin (Pdpn) (23), which is highly expressed in CD11c+ conventional DCs (cDCs) as well as that of Pdpn+ FRCs and T cells in the adult mouse spleen. Such ablation also impaired the survival of FRCs as well as the production by CD4+ cDCs of tumor necrosis factor receptor (TNFR) ligands, including TNF-α, which was shown to promote the proliferation and survival of Pdpn+ FRCs. CD4+ cDCs thus regulate the steady-state homeostasis of FRCs in the adult spleen via the production of TNFR ligands, with the CD47–SIRPα interaction in cDCs likely being indispensable for such regulation. This article contains supporting information online at www.pnas.org/cgi/doi/10.1073/pnas.1711345114/PNASPlus.

Significance

Homeostasis of Pdpn+ fibroblastic reticular cells (FRCs) is thought to be regulated by hematopoietic cells. However, the cellular and molecular mechanisms of such homeostasis have been poorly understood, especially under the steady-state condition in adults. We show that dendritic cells, particularly CD4+ conventional dendritic cells (cDCs), are crucial for the homeostasis of FRCs in the adult spleen under the steady-state condition. The production of TNF receptor ligands by CD4+ cDCs regulates such homeostasis of FRCs, with SIRPα and CD47 likely being indispensable for the production of TNFR ligands by CD4+ cDCs. The CD47–SIRPα interaction on CD4+ cDCs may thus control the homeostasis of FRCs, which in turn maintain homeostasis of T cells in the white pulp of the spleen.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/doi/10.1073/pnas.1711345114/DCSSupplemental.
protein of the Ig superfamily that is expressed ubiquitously. We have previously shown that both SIRPα-deficient mice and mice that express a mutant form of SIRPα lacking the cytoplasmic region manifest a marked reduction in the number of CD4+ cDCs in the spleen and PPs (14, 17). Such SIRPα-mutant mice also manifested a reduction in the number of T cells and podoplanin (Pdpn)+ FRCs in the spleen (18), suggesting that SIRPα is important for homeostasis of CD4+ cDC subsets as well as of T cells and FRCs in SLOs. The cellular and molecular basis for such regulation by SIRPα has remained largely unknown, however. To characterize such regulation, we have generated and analyzed DC-specific SIRPα or CD47 conditional-knockout mice.

Results
Importance of SIRPα on DCs for Homeostasis of CD4+ cDCs, T Cells, and FRCs in the Spleen. We recently generated mice in which SIRPα is specifically ablated in CD11c+ DCs (SirpaΔDC mice) (17). As we previously reported, we confirmed that SirpaΔDC mice (at 8–12 wk of age) manifested a marked reduction specifically in the population of CD4+ cDCs in the spleen (Fig. 1A) and in peripheral LNs (17) compared with control Sirpa+ mice. We also found that SIRPα expression was partially down-regulated in both CD11c+CD11b+Ly6C+ monocytes and CD11c+CD11b+Ly6G+ granulocytes in the spleens of SirpaΔDC mice (Fig. S1A) (19). In contrast to CD4+ cDCs, the number of Ly6G+ monocytes was increased in the spleens of SirpaΔDC mice compared with control mice (Fig. S1B). In addition, H&E staining revealed that the white pulp of the spleen was significantly smaller in SirpaΔDC mice than in Sirpa+ mice (Fig. 1B). Quantitative immunohistochemistry analysis of T cells (Thy1.2+) and B cells (B220+) in the spleens of adult SirpaΔDC mice revealed that the area of the T cell zone, but not that of the B cell zone, was indeed reduced (Fig. 1C). Consistent with this finding, the frequency of T cells and the absolute numbers of both CD4+ and CD8+ T cells were markedly reduced in the spleens of SirpaΔDC mice compared with control mice (Fig. 1D). By contrast, the frequency and absolute numbers of B cells were not reduced in the spleens of SirpaΔDC mice (Fig. 1D). Furthermore, the absolute numbers of T and B cells in (Fig. S2 A–C) peripheral LNs, mesenteric LNs, or Peyer’s patch did not differ between SirpaΔDC and Sirpa+ mice. These results thus suggested that SIRPα on DCs is an important determinant of the abundance of CD4+ cDCs and T cells in the spleen.

We next examined the population of FRCs in the spleens of SirpaΔDC mice. With the use of flow cytometry, CD45+ Ter119− splenic SCs were segregated into three subsets on the basis of surface expression of Pdpn and Cd31 (20): Pdpn+ Cd31− (FRCs), Pdpn− Cd31− (blood endothelial cells, or BECs), and Pdpn+ Cd31− (blood endothelial cells, or BECs), and Pdpn− Cd31− (blood endothelial cells, or BECs), and Pdpn+ Cd31− (blood endothelial cells, or BECs).

Fig. 1. Importance of SIRPs on DCs for homeostasis of CD4+ cDCs, T cells, and FRCs in the spleen. (A) Splenocytes isolated from Sirpa+ or SirpaΔDC mice at 8–12 wk of age were analyzed for cDC subsets by flow cytometry. Representative plots for CD4+ (CD4+CD8−), CD8+ (CD8+CD4−), and DN (CD4−CD8−) subsets among CD11c+CD11b+ cDCs (Left) and the absolute numbers of these cells (Right) are shown. (B, Left) Paraffin-embedded sections of the spleen from Sirpa+ or SirpaΔDC mice were stained with H&E. Dashed lines demarcate white pulp area. (Right) The size of the white pulp area was determined as the percentage of total spleen area with the use of ImageJ software. (C) Upper) Frozen sections of the spleen from Sirpa+ or SirpaΔDC mice were stained with antibodies to Thy1.2 (red) and B220 (green). (Scale bar, 500 μm.) (Lower) The Thy1.2+ or B220+ area in each image was measured with the use of ImageJ software. (D) Splenocytes isolated from Sirpa+ or SirpaΔDC mice were stained for flow cytometric determination of the frequency of T cells (CD3+) and B cells (B220+) among total splenocytes (Left) or of the absolute numbers of B cells, CD4+ T cells (CD3+CD4+), and CD8+ T cells (CD3+CD8+) (Right). (E) Splenocytes isolated from Sirpa+ or SirpaΔDC mice were analyzed for SC subsets by flow cytometry. Representative plots for FRCs (Cd31+Pdpn+) and BECs (Cd31−Pdpn+) among Ter119−CD45− nonhemopoietic cells (Left) and the absolute numbers of these cells (Right) are shown. SSC, side scatter. (F) Frozen sections of the spleen from Sirpa+ or SirpaΔDC mice were stained with antibodies to Pdpn (red) and to B220 (green) (Left), and the Pdpn+ area was measured in each image (Right). (G) Relative Cd19, Cd21, and Ilt7 mRNA abundance in the spleens of Sirpa+ or SirpaΔDC mice. The amount of each target mRNA was normalized by that of Gapdh mRNA and expressed relative to the value for Sirpa+ mice. (H) Frozen sections of the spleen from Sirpa+ or SirpaΔDC mice were stained for Ccl21 (red) and B220 (green) (Left), and the Ccl21+ area was quantified in each image (Right). (Scale bar, 500 μm.) All quantitative data are pooled from three independent experiments and are expressed as means ± SE for three (A and E), five (B, C, F, and H), six (G), or eight (D) mice per group. *P < 0.05, **P < 0.01, ***P < 0.001 (Student’s t test).
cells (Fig. 1E). The absolute number of FRCs was significantly reduced in the spleens of Sirpa$^\Delta DC$ mice compared with that of Sirpa$^+$ mice (Fig. 1E). Consistent with this finding, immunohistofluorescence analysis showed that staining for Pdpn, which identifies FRCs in the T cell zone (21, 22), was also markedly reduced in the spleens of Sirpa$^\Delta DC$ mice (Fig. 1F). By contrast, the absolute number of FRCs in peripheral LNs, mesenteric LNs, or Peyer’s patch did not differ between Sirpa$^\Delta DC$ and Sirpa$^+$ mice (Fig. S2 D–F).

The homing and survival of T cells in SLOs are thought to be supported by CCL19, CCL21, and IL-7, all of which are produced by FRCs (4, 5), whereas the homing of B cells is supported by CXCL13 produced by FDCs (1). The abundance of Ccl19, Ccl21, and Il7 mRNAs in the spleen was significantly reduced in Sirpa$^\Delta DC$ mice compared with Sirpa$^+$ mice (Fig. 1G). Moreover, immunohistofluorescence analysis revealed a markedly reduced level of CCL21 staining in the T cell zone of Sirpa$^\Delta DC$ mice (Fig. 1H), whereas CXCL13 staining in B cell follicles did not differ between Sirpa$^\Delta DC$ and Sirpa$^+$ mice (Fig. S2G).

**Increased Turnover and Apoptosis of FRCs in the Spleens of Sirpa$^\Delta DC$ Mice.** We next examined the turnover of splenic SC subpopulations by monitoring the kinetics of cell labeling with BrdU in the continuous presence of this agent (14). Whereas 9.7 ± 5.0% (mean ± SE) of FRCs in the spleens of Sirpa$^+$ mice were labeled with BrdU at 6 d after exposure to this agent, 33.3 ± 7.0% of those in the spleens of Sirpa$^\Delta DC$ mice were so labeled (Fig. 2 A). Analysis of cell-cycle status for SCs by staining with antibodies to Ki-67 and Hoechst 33342 similarly revealed that the proportion of quiescent FRCs in G0 phase was markedly reduced, whereas that of interphase FRCs in G1 or S-G2-M was increased in the spleens of Sirpa$^\Delta DC$ mice (G0, 16.2 ± 4.0%; G1, 32.8 ± 1.5%; S-G2-M, 42.4 ± 5.7%) compared with the proportions in Sirpa$^+$ mice (G0, 68.8 ± 8.0%; G1, 10.5 ± 2.6%; S-G2-M, 9.2 ± 6.7%) (Fig. 2B). These data suggested that the turnover rate of FRCs was markedly increased in the spleens of Sirpa$^\Delta DC$ mice. We further examined the frequency of apoptotic cells by staining with Annexin V. The frequency of Annexin V$^+$ cells among total FRCs in the spleen was greatly increased in Sirpa$^\Delta DC$ mice compared with Sirpa$^+$ mice (77.8 ± 6.0% versus 16.8 ± 1.9%) (Fig. 2C), suggesting that the deficiency of FRCs in the spleens of Sirpa$^\Delta DC$ mice is attributable, at least in part, to reduced survival of these cells.

**Importance of Sirpα on DCs for Homeostasis of FRCs in the Adult Spleen.** We next examined whether the reduction in the number of FRCs in the spleens of Sirpa$^\Delta DC$ mice was also apparent during the early stages of postnatal development. Whereas a significant difference in the Pdpn$^+$ area in the spleens of Sirpa$^\Delta DC$ mice and Sirpa$^+$ mice could not be demonstrated at 1 and 2 wk after birth, the area was markedly diminished in Sirpa$^\Delta DC$ mice at 3 wk (Fig. S3 A and B). To investigate further the importance of SirpαRα on DCs for homeostasis of CD4$^+$ cDCs and FRCs in the adult spleen under the steady-state condition, we crossed Sirpα$^+$ mice with ROSA26-CreER$^{T2}$ mice (23) to generate tamoxifen (TAM)-inducible SirpαRα-knockout (SirpaRα iKO) mice. Sirpα iKO and control Sirpα$^+$ mice were injected s.c. with TAM on days −4, −2, 0 and were analyzed on days 3, 7, and 21 (Fig. 3A). On day 3, both the expression of SirpαRα on splenic CD4$^+$ cDCs (Fig. 3B) and the frequency of CD4$^+$ cDCs in the spleen (Fig. 3C) were markedly reduced in SirpaRα iKO mice compared with control mice. By contrast, the size of the Pdpn$^+$ area in the spleens of SirpaRα iKO mice was similar to that in Sirpa$^+$ mice at this time (Fig. 3 D and E). At day 7, however, the size of the Pdpn$^+$ area was significantly reduced in SirpaRα iKO mice (Fig. 3 D and E). Furthermore, although a reduction in T cell frequency was not apparent in the spleens of SirpaRα iKO mice at day 7, it was evident at day 21 (Fig. 3F). The area occupied by T cells was also reduced at day 21, whereas that occupied by B cells was not altered (Fig. S3C). These results suggested that SirpαRα on DCs is important for homeostatic regulation of CD4$^+$ cDCs as well as T cells and FRCs in the spleen in adulthood. They also indicated that the reduction in the number of CD4$^+$ cDCs induced by SirpαRα ablation in DCs precedes the reduction in FRCs, which in turn precedes the reduction in T cells.

**Loss of FRCs in the Spleens of Sirpa$^\Delta DC$ Mice Is Likely Due to a Functional Defect of CD4$^+$ cDCs.** The deficiency of FRCs and T cells in the spleens of Sirpa$^\Delta DC$ mice was likely attributable to the ablation of SirpαRα specifically in DCs. We therefore next examined whether the loss of these cells is due to the reduction in the number of CD4$^+$ cDCs or to a functional defect of cDCs. We first generated bone marrow (BM) chimera by transferring individual or mixed (1:1 ratio) BM from Sirpa$^\Delta DC$ (CD45.2$^+$) and WT (CD45.1$^+$) mice into lethally irradiated WT (CD45.1$^+$CD45.2$^-$) recipients. Eight weeks after reconstitution, the resulting WT:Sirpa$^\Delta DC$ (1:1) mixed

**Fig. 2.** Increased turnover and apoptosis of FRCs in the spleens of Sirpa$^\Delta DC$ mice. (A, Left) Sirpa$^{+/+}$ and Sirpa$^{\Delta DC}$ mice were injected i.p. with 1 mg of BrdU and then were continuously supplied with BrdU (0.8 mg/ml) in drinking water for 6 d. Then staining for BrdU among BECs and FRCs in the spleens of Sirpa$^{+/+}$ or Sirpa$^{\Delta DC}$ mice (treated open traces) or untreated (filled traces) with BrdU was determined by flow cytometry. (Right) The percentage of BrdU$^+$ cells among BECs and FRCs was also determined. Data are pooled from three independent experiments and are expressed as means ± SE for six mice per group. *P < 0.05 (Student’s t test), (B) Representative flow cytometric profiles for G0 (Ki-67$^+$Hoechst 33342$^-$), G1 (Ki-67$^-$/Hoechst 33342$^+$), and S-G2-M (Ki-67$^-$/Hoechst 33342$^+$) cells (Left) and the proportion of cells in G0, G1, or S-G2-M phases (Right) among BECs and FRCs in the spleens of Sirpa$^{+/+}$ or Sirpa$^{\Delta DC}$ mice. Data are pooled from three independent experiments and are expressed as means ± SE for three mice per group. *P < 0.05, **P < 0.01 (two-way ANOVA and Sidak’s test). (C) Representative flow cytometric profiles for Annexin V$^+$ cells (Left) and the percentage of Annexin V$^+$ cells (Right), among BECs and FRCs in the spleens of Sirpa$^{+/+}$ or Sirpa$^{\Delta DC}$ mice. Data are pooled from three independent experiments and are expressed as means ± SE for four mice per group. **P < 0.01 (Student’s t test).
BM → WT chimeras manifested significant recovery of CD4+ cDCs in the spleen compared with the Sirpa−/−DC BM (single) → WT chimeras (Fig. 4A). However, the number of CD4+ cDCs in the former chimeras was much smaller than in the control Sirpa+/+ BM → WT chimeras. Moreover, 81.7 ± 1.9% (mean ± SE) of the CD4+ cDCs in WT:Sirpa+/+ (1:1) mixed BM → WT chimeras were derived from CD45.1+ WT mice (Fig. S4A). These results were thus consistent with the notion that SIRPα in cDCs is intrinsically important for homeostasis of CD4+ cDCs in the spleen. In contrast, the Pdpn+ area and the T cell area in the spleens of WT:Sirpa+/+ (1:1) mixed BM → WT chimeras was much greater than in Sirpa−/−DC BM (single) → WT chimeras and was almost equivalent to control Sirpa+/+ BM → WT chimeras (Fig. 4B and Fig. S4B), suggesting that the reduction in the number of FRCs, as well as the reduction in the number of T cells, in the spleens of Sirpa−/−DC mice is attributable to a functional defect of, rather than to a reduced abundance of, CD4+ cDCs.

We next examined spleens from DC-specific Irf4-deficient (Irf4ΔDC) or DC-specific Rbpj-deficient (RbpjΔDC) mice, both of which were previously found to manifest a selective reduction in the number of CD4+ cDCs in the spleen (24, 25). The extent of the selective reduction in the CD4+ cDC population in Irf4ΔDC or RbpjΔDC mice (Fig. 4C) was similar to that in Sirpa−/−DC mice (Fig. 1A). However, the size of the Pdpn+ area and of the T cell zone in the spleen did not differ significantly between Irf4ΔDC or RbpjΔDC mice and their corresponding control mice (Fig. 4D and E and Fig. S4 C and D). These results thus further suggested that the reduction in the number of FRCs and the reduction in the number of T cells, in the spleens of Sirpa−/−DC mice is attributable to a functional defect in the corresponding CD4+ cDCs.

**SIRPα+ cDCs Promote Proliferation or Survival of Splenic FRCs via TNF Receptor Ligands in Vitro.** We next examined whether DCs are able to promote the proliferation or survival of SCs in vitro. To this end, we developed a coculture system for DCs and splenic SCs. The cultured SCs, isolated as CD45− cells from the spleens of WT mice (26), manifested a spindle-shaped morphology and expressed Pdpn at a high level but were negative for CD31 (Fig. 5A and B). They also expressed both TNF receptor 1 (TNFR1) and LTβR. Stimulation of these SCs with recombinant TNF-α or an agonistic monoclonal antibody to LTβR induced activation of canonical or noncanonical NF-κB signaling pathways (27, 28), respectively, as evidenced by the degradation of either the endogenous NF-κB inhibitor IκBα or NF-κB2 (p100), respectively (Fig. 5D). Furthermore, both recombinant TNF-α and anti-LTβR significantly promoted the proliferation of these SCs (Fig. 5E), with the effect of TNF-α being more pronounced than that of anti-LTβR. The cultured splenic CD45+ SCs were thus phenotypically and functionally similar to FRCs, as described previously (22, 28).

Coculture of the splenic CD45+ SCs from WT mice with splenic CD11c+ DCs from Sirpa−/− mice in medium containing 10% FBS induced a threefold increase in the extent of proliferation of the former cells (Fig. 5F). By contrast, DCs from Sirpa+/+ mice had a markedly smaller effect on the proliferation of SCs than that of DCs from Sirpa−/− mice (Fig. 5F). In addition, we found that the number of CD45+ cells harvested from the coculture of CD11c+ cells with CD45+ SCs was similar between Sirpa−/− and Sirpa+/+ mice (Fig. 5F). Monitoring of the number of live CD45+ splenic SCs in medium containing 0.5% FBS revealed that the survival of these cells cocultured with Sirpa+/+ DCs was much greater than that of those cultured without DCs or those cocultured with Sirpa−/−DCs (Fig. 5G).

To examine whether direct contact of DCs with SCs is required for the effect of DCs on the proliferation or survival of SCs, we cultured the two cell types in separate chambers of a Transwell plate (filter pore size, 0.4 μm). The proliferation of splenic SCs placed in the lower chamber was promoted by the presence of Sirpa−/−DCs in the upper chamber to an extent similar to that observed in nonseparated cocultures (Fig. 5H). By contrast, the effect of Sirpa−/−DCs placed in the upper chamber was minimal (Fig. 5H). These results suggested that direct contact of DCs with SCs is not required for the effect of DCs on the proliferation or

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**Fig. 3.** Importance of SIRPα on DCs for homeostasis of splenic FRCs in adulthood. (A) Sirpa−/− and Sirpa iKO mice were injected s.c. with TAM on days −4, −2, and 0. The spleen was isolated on days 3, 7, or 21 for immunohistofluorescence or flow cytometric analysis. (B) Staining for SIRPα (open traces) or with an isotype control antibody (filled traces) on CD45− cDCs isolated from the spleens of Sirpa iKO mice 3 d after the last TAM ([TAM]+) or vehicle ([TAM]−) injection. Data are representative of three mice. (C) Representative flow cytometric profiles for CD8+, CD4+, and DN cDC subsets (left) and the percentage of CD4+ cDCs (right) in the spleens of Sirpa−/− or Sirpa iKO mice at 3, 7, and 21 d after the last TAM injection. (D) Frozen sections of spleens from Sirpa−/− and Sirpa iKO mice at 3, 7, and 21 d after the last TAM injection were stained with antibodies to Pdpn (red) and to B220 (green). (Scale bar, 500 μm.) (E) The Pdpn+ area in the spleens of Sirpa−/− and Sirpa iKO mice at 3, 7, and 21 d after the last TAM injection was measured in images similar to those in D with the use of ImageJ software. (F) The percentage of T cells among total splenocytes of Sirpa−/− and Sirpa iKO mice at 3, 7, and 21 d after the last TAM injection was determined by flow cytometry. All quantitative data (C, E, and F) are pooled from three independent experiments and represent the means ± SE for three mice per group. *P < 0.05, **P < 0.01 (Student’s t test).
survival of SCs; the effect likely is mediated by a factor produced and secreted by DCs. Indeed, culture supernatants harvested from cocultures of \textit{Sirpa}\textsuperscript{f/f} DCs and SCs after 5 d markedly promoted the proliferation of freshly prepared SCs, whereas the effect of culture supernatants from cocultures of \textit{Sirpa}\textsuperscript{Δ}\textsubscript{DC} DCs and SCs was again minimal (Fig. 5I).

Both TNFR ligands (such as TNF-α or a soluble form of LT\textsubscript{α}) and LT\textsubscript{αβ} ligands (such as the membrane-anchored form of LT\textsubscript{αβ}1) are thought to be important for the proliferation and survival of FRCs in SLOs (22, 29). We therefore examined whether such cytokines contribute to the effect of DCs on the proliferation of SCs in the coculture system. A p55 TNFR (TNFR1)-Fc chimera, which is thought to neutralize TNFR ligands (30), markedly inhibited the stimulation of SC proliferation by cocultured \textit{Sirpa}\textsuperscript{Δ}\textsubscript{DC} DCs, whereas an LT\textsubscript{αβ}R-Fc chimera that neutralizes membrane-anchored LT\textsubscript{αβ}R ligands (30) had no such effect (Fig. 5J), suggesting that TNFR ligands are important for this stimulatory action of DCs. Neither Fc fusion protein inhibited the effect of \textit{Sirpa}\textsuperscript{Δ}\textsubscript{DC} DCs on the proliferation of cocultured SCs (Fig. 5J). We indeed detected TNF-α in culture supernatants of cocultured \textit{Sirpa}\textsuperscript{Δ}\textsubscript{DC} DCs and SCs, whereas the amount of TNF-α in the culture supernatants of cocultured \textit{Sirpa}\textsuperscript{Δ}\textsubscript{DC} DCs and SCs was much less (Fig. 5K).

Fig. 4. Loss of FRCs in the spleens of \textit{Sirpa}\textsuperscript{Δ}\textsubscript{DC} mice is likely due to a functional defect in CD4\textsuperscript{+} cDCs. (A) Lethally irradiated WT (CD45.1\textsuperscript{+}CD45.2\textsuperscript{+}) mice were reconstituted with BM cells from \textit{Sirpa}\textsuperscript{f/f} or \textit{Sirpa}\textsuperscript{Δ}\textsubscript{DC} mice (CD45.2\textsuperscript{+}) or with an equal mixture of BM cells from \textit{Sirpa}\textsuperscript{Δ}\textsubscript{DC} and WT (CD45.1\textsuperscript{+}) mice to generate \textit{Sirpa}\textsuperscript{f/f}, \textit{Sirpa}\textsuperscript{Δ}\textsubscript{DC} (single), or \textit{Sirpa}\textsuperscript{Δ}\textsubscript{DC} + WT (1:1) BM chimeras, respectively. Eight weeks after cell transplantation, the spleens from the BM chimeras were isolated for analysis. Representative flow cytometric profiles for CD8\textsuperscript{+}, CD4\textsuperscript{+}, or DN cDC subsets (Left) and the percentages of these cells among total splenocytes (Right) are shown. (B, Left) Frozen sections of the spleens of BM chimeras were stained with antibodies to Pdpn (red) and to B220 (green). (Scale bar, 200 \textmu m.) (Right) The Pdpn\textsuperscript{+} area was measured in each image with the use of ImageJ software. (C) Representative flow cytometric profiles for CD4\textsuperscript{+}, CD8\textsuperscript{+}, and DN cDCs in the spleens of \textit{irf4}\textsuperscript{f/f} and \textit{irf4}\textsuperscript{Δ}\textsubscript{DC} mice (Upper Left) or \textit{Rbpj}\textsuperscript{f/f} and \textit{Rbpj}\textsuperscript{Δ}\textsubscript{DC} mice (Lower Left) and the corresponding absolute numbers of these cells (Right) are shown. (D and E) Frozen sections of the spleens from \textit{irf4}\textsuperscript{Δ}\textsubscript{DC} and \textit{irf4}\textsuperscript{f/f} mice (D, Left) or from \textit{Rbpj}\textsuperscript{Δ}\textsubscript{DC} and \textit{Rbpj}\textsuperscript{f/f} mice (E, Left) were stained with antibodies to Pdpn (red) and to B220 (green). (Scale bars, 500 \textmu m.) (D, Right and E, Right) The Pdpn\textsuperscript{+} area in each image was also measured. Data are pooled from three independent experiments and are expressed as the means ± SE for four (A and B) or three (C–E) mice per group. *P < 0.05, **P < 0.001 by one-way ANOVA and Tukey’s test (A and B) or by Student’s t test (C); ns, not significant.
contrast, we did not detect LTα in the supernatants of either type of coculture (Fig. S5B). These results thus suggested that DCs promote the proliferation or survival of SCs through the secretion of soluble TNFR ligands such as TNF-α and that Sirpα on DCs is important for this function of DCs.

Importance of TNFR Ligands for Regulation by DCs of FRC Homeostasis in the Spleen in Vivo. We measured the abundance of mRNAs for TNF-α and LT in cDCs of the spleen. Among the three cDC subsets, the amounts of Tnf, Lta, and Ltb mRNAs were highest in CD4+ cDCs (Fig. 6A), which also express Sirpα at the highest level (31). Indeed, the ability of CD4+ cDCs to promote the proliferation of splenic SCs in coculture was much greater than that of CD8+ or double-negative (DN) cDCs (Fig. 6B). In addition, this ability of CD4+ cDCs was inhibited by the TNFR-Fc chimera (Fig. 6C). Moreover, the expression of Tnf, Lta, and, to a lesser extent, Ltb was down-regulated in splenic CD4+ cDCs isolated from Sirpα<sup>ADC</sup> mice compared with those from Sirpα<sup>sh</sup> mice (Fig. 6D). In addition to TNF family cytokines, the amount of IL1β was also specifically down-regulated in splenic CD4<sup>+</sup> cDCs isolated from Sirpα<sup>ADC</sup> mice (Fig. S6A), suggesting that the defect in cytokine production from Sirpα<sup>ADC</sup> cDCs was not specific to the TNF family. To further demonstrate the impact of TNFR ligands derived from CD4<sup>+</sup> cDCs in vivo, we isolated CD4<sup>+</sup> cDCs from WT (CD45.1<sup>+</sup>) or Sirpα<sup>ADC</sup> (CD45.2<sup>+</sup>) mice in the spleens of WT-Sirpα<sup>ΔLκ</sup> (1:1) mixed BM or single → WT chimeras (Fig. S6B). The expression of Tnf mRNA in the spleens of mixed chimeras was specifically impaired in CD4<sup>+</sup> cDCs derived from Sirpα<sup>ADC</sup> donors, to the same extent as those derived from Sirpα<sup>ADC</sup> BM (single) → WT chimeras, compared with that from WT donors (Fig. S6C). In contrast, the expression of Tnf, Lta, and Ltb mRNAs was not reduced in Ly6C<sup>hi</sup> monocytes from Sirpα<sup>ADC</sup> BM (WT→WT) chimeras compared with that of WT donors.
mice compared with those from Sirpa<sup>ΔDC</sup> mice (Fig. S6D). These results thus suggested that SIRPα-deficient CD4<sup>+</sup> cDCs are functionally defective with regard to the production of TNFR ligands.

We next examined whether TNFR ligands indeed participate in the regulation of FRC homeostasis in the spleen in vivo. To this end, we treated mice with a human p75 TNFR-Ig fusion protein (Eta-nercept) that is able to bind to and neutralize mouse TNF-α and LTN(IF-32). Treatment of Sirpa<sup>ΔDC</sup> mice with Eta-nercept resulted in a marked reduction in the size of the Pdpn<sup>+</sup> area and in the frequency of FRCs in the spleen (Fig. 6 D and E). In contrast, Eta-nercept did not affect the already reduced size of the Pdpn<sup>+</sup> area in the spleens of Sirpa<sup>ΔDC</sup> mice (Fig. 6D). Moreover, the number of Annexin V<sup>+</sup> FRCs was significantly increased in the spleens of Sirpa<sup>ΔDC</sup> mice treated with Eta-nercept (Fig. 6F). By contrast, treatment of Sirpa<sup>ΔF</sup> mice with Eta-nercept had no effect on the number of CD4<sup>+</sup> or other cDCs and resulted in a slight (but not significant) reduction of T cells in the spleen compared with PBS-treated mice (Fig. 6G). Indeed, the size of the T zone (as well as the number of T cells) was not markedly changed (32, 33), but the T cell homing was impaired (12) in the spleens of TNF-α-KO mice. Taken together, these results suggested that TNFR ligands (such as TNF-α and LTRα), which are likely derived from SIRPα-expressing CD4<sup>+</sup> cDCs, are important for FRC homeostasis in the spleen in vivo.

LTi cells play a central role in the development of lymphoid organs during mouse embryogenesis (34) and are also thought to be present in and to be important for maintenance of the structural organization of SLOs in adulthood (35, 36). However, the frequency of CD11c<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup> LTi cells in the spleen was found not to differ between Sirpa<sup>ΔF</sup> and Sirpa<sup>ΔDC</sup> mice (Fig. 6H).

**Importance of CD47 on DCs for Homeostasis of CD4<sup>+</sup> cDCs, T Cells, and FRCs in the Spleen.** To investigate the mechanism by which SIRPα promotes the function of cDCs in regulating FRC homeostasis, we examined the possible role of the SIRPα ligand CD47 expressed on DCs in such regulation. Given that CD47<sup>+</sup> is expressed ubiquitously, we first generated CD47<sup>−/−</sup> mice in which exon 1 of the CD47 gene is flanked by two loxP sites (Fig. S7A). DC-specific CD47-deficient (CD47<sup>ΔDC</sup>) and systemic CD47-deficient (CD47<sup>Δs</sup>) mice were then generated by crossing CD47<sup>−/−</sup> mice with CD11c-Cre or E2A-Cre mice, respectively. We confirmed that CD47 expression was lost specifically in cDCs and plasmacytoid DCs (pDCs) in the spleens of CD47<sup>ΔDC</sup> mice (Fig. S7B). The number of cDCs—in particular, the number of CD4<sup>+</sup> cDCs—was greatly reduced in the spleens of CD47<sup>ΔDC</sup> mice compared with CD47<sup>+/+</sup> mice (Fig. 7A). Furthermore, the numbers of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fig. 7B) and the size of the T cell zone (Fig. 7C) were significantly
reduced in the spleens of CD47ΔDC mice. The area occupied by Pdpn+ FRCs also was reduced in the spleens of CD47ΔDC mice (Fig. 7D). CD47ΔDC mice thus appeared to be a phenocopy of SirpaΔDC mice in terms of the number of cDCs, T cells, and Pdpn+ FRCs in the spleen. The number of CD4+ cDCs also was reduced in peripheral LNs of CD47ΔDC mice, whereas the absolute number of FRCs was not reduced in these LNs of CD47ΔDC mice compared with those of CD47+/+ mice (Fig. S7 C and D).

To examine further the importance of CD47 on DCs for regulation of FRC homeostasis, we cocultured CD11c+ cells isolated from the spleens of CD47ΔDC or CD47+/+ mice with splenic SCs of WT mice. Coculture with CD47ΔDC DCs resulted in...
in marked inhibition of both the proliferation (Fig. 7E) and survival (Fig. 7F) of SCs compared with those apparent on coculture with CD47ΔCDc DCs, suggesting that CD47 on DCs is indeed important for homeostasis of DCs and FRCs in the spleen. We also examined the impact of CD47 ablation in splenic SCs on their proliferation in vitro. The ability of CD47-deficient SCs (isolated from CD47ΔCDc mice) to proliferate on coculture with CD47ΔCDc DCs was similar to that of CD47ΔCDc SCs (Fig. 7G), suggesting that CD47 in SCs is dispensable for the proliferation of these cells in the presence of DCs. Furthermore, the abundance of Tnf, Ltu, and Lib mRNAs was greatly reduced in splenic CD4+ cDCs isolated from CD47ΔCDc mice compared with those from CD47f/f mice (Fig. 7H).

**Discussion**

We have here shown that ablation of SIRPs in cDCs results in a marked reduction in the numbers of CD4+ cDCs, Pdpn+ FRCs, and T cells in the adult spleen. A similar reduction in the number of CD4+ cDCs, but not of FRCs or T cells, was also apparent in the peripheral LNs of SirpaΔDCc mice. Loss of FRCs was likely responsible for the observed down-regulation of the splenic expression of CCL19, CCL21, and IL-7, all of which are produced by FRCs and attract or support T cells in white pulp, with this down-regulation in turn resulting in the loss of T cells in the spleens of SirpaΔDCc mice. In addition, the reduction in the number of CD4+ cDCs induced by TAM injection in Sirpa ΔKO mice was followed by the consecutive depletion of FRCs and T cells in the spleen, suggesting that the loss of FRCs is attributable to either a reduced abundance of or a functional defect in CD4+ cDCs. Transfer of a 1:1 mixture of BM from WT and Sirpa+/- mice into irradiated recipient animals resulted in a substantial recovery of DCs but not of CD4+ cDCs. In addition, both Irf4+DC and Rhy+DCc mice manifested a marked reduction in the number of CD4+ cDCs without any reduction in the number of FRCs or T cells in the spleen. Furthermore, DCs isolated from the spleens of Rhy+ΔCDc mice supported the proliferation of splenic SCs to an extent similar to that observed with DCs from control mice (Fig. S8). Together, these results indicate that a functional defect in CD4+ cDCs induced by SIRPs deficiency is responsible for the reduction in the number of FRCs in the spleens of SirpaΔDCc mice. In addition, other cDC subsets, such as DN cDCs, likely compensate for the lack of CD4+ cDCs of Rhy+ΔCDc and perhaps Irf4+ΔCDc mice with regard to the maintenance of FRCs in the spleen.

With the use of our coculture system for DCs and splenic SCs, we found that DCs—in particular, CD4+ cDCs, which, among cDC subsets, express Tnf, Ltu, and Lib at the highest levels—promoted the proliferation or survival of CD45+ SCs in vitro. Culture in a Transwell plate or in the presence of an antagonist of TNFR ligands showed that SIRPα+CD4+ cDCs likely promote the proliferation or survival of FRCs by producing TNFR ligands such as TNF-α. Indeed, the expression of Tnf and Ltu was down-regulated in CD4+ cDCs from SirpaΔDCc mice, whose ability to support the proliferation or survival of FRCs was greatly diminished. We further showed that treatment of mice with Etanercept, which neutralizes TNF-α or LTR2, resulted in a marked reduction in the size of the FRC area in the spleen. Together, these observations indicate that the production of TNFR ligands by CD4+ cDCs regulates homeostasis of FRCs in the adult spleen under the steady-state condition, with SIRPα likely being indispensable for the production of such ligands by CD4+ cDCs. By contrast, Etanercept had no effect on the number of CD4+ cDCs and had minimal effect on the number of T cells, although we noted a significant reduction of FRCs in the spleen. Such discrepancy between the number of T cells and the number of FRCs may be attributable to the duration of Etanercept treatment or the maintenance of T cell survival by DCs through the presentation of self-antigens (37). The expression of TNF-α in DN cDCs was not high but was down-regulated in the spleens of SirpaΔDCc mice, as was the expression in CD4+ cDCs. Thus, the FRC phenotype in SirpaΔDCc mice is likely attributable, in part, to a reduced production of TNF-α from DN cDCs. Consistent with this notion, TnfΔ− or TnfΔ+/− mice manifest marked down-regulation of CCL21 or CCL19 expression in the spleen (11, 12), with the former mice also manifesting poor development of Pdpn+ FRCs in the spleen but not in LNs (12). Given that DCs in peripheral LNs promote the survival of FRCs through the action of the LTβR ligands LTα2β1 or LIGH, under inflammatory conditions (29), the mechanism by which DCs regulate FRCs likely differs between the spleen and LNs or between noninflammatory and inflammatory conditions.

Generation or homeostasis of SCs is thought to require various types of hematopoietic cells in neonates as well as in adult mice. Indeed, splenic B cells were shown to regulate FRC development and maintenance in a manner dependent on LTR2 expression in B cells (10). LTI cells are also thought to contribute to the restoration of FRCs after lymphocytic choriomeningitis virus infection through the action of LTR2β1-expressed on the LTI cells (36). We now have shown that the reduction in the number of Pdpn+ FRCs in the spleens of SirpaΔDCc mice was already pronounced by 3 wk of age. Consistent with this finding, CD4+ cDCs increase in number and become predominant among the cDC subsets in the spleen by 3 wk of age (38). We thus have shown that DCs, particularly CD4+ cDCs, play an important role in the homeostasis of FRCs in the spleen under the steady-state condition in adulthood.

We found that CD47 on DCs is also important for maintaining the abundance of CD4+ cDCs, Pdpn+ FRCs, and T cells in the spleen. In addition, CD47 was found to be indispensable for DCs’ ability to promote the proliferation of FRCs in vitro and for the expression of TNFR ligand genes in CD4+ cDCs. These results suggest that interaction of CD47 with SIRPα either in cis within individual cDCs or in trans between neighboring cDCs (39) is important for the production of TNFR ligands by CD4+ cDCs. In particular, the trans-interaction between CD47 [on non-hematopoietic cells (14) or red blood cells (40)] and SIRPα on DCs is implicated in the homeostasis of CD4+ cDCs in the spleen. Ligation of SIRPα by CD47 [likely in trans] induces the tyrosine phosphorylation and activation of the tyrosine phosphatases Shp1 and Shp2 (13). In addition, Shp2 is a positive regulator of the...
FcRRI-induced activation of the Fyn and Ras–MAPK signaling pathways that lead to the release of TNF-α from mast cells (41). However, further study is required to characterize the mechanism by which SIRPα through its interaction with CD47 promotes the production of TNFR ligands by cDCs in the spleen.

In summary, we propose a model for the role of CD47–SIRPα interaction in the regulation by cDCs of the FRC network in the adult spleen (Fig. 8). Given that the CD47–SIRPα system plays an important role in the regulation of autoimmunity and protection from pathogens (42), further study will be required to determine whether SIRPα in DCs also regulates the organization and regeneration of SLOs during inflammation. In addition, our findings may be relevant to anti-TNF therapy with Etanercept, with such treatment having been shown to repress adaptive immune responses in patients with rheumatoid arthritis. Regulation of the FRC network thus might contribute, at least in part, to the efficacy of this treatment.

Materials and Methods

Antibodies and reagents, generation of CD47Δα mice, cell preparation and flow cytometry, isolation and culture of splenic SCs, determination of BrdU incorporation, analysis of cell-cycle profile and apoptosis, staining, immunohistofluorescence analysis, cell sorting of cDC subsets and monocytes, preparation of cDNA and RT-qPCR analysis, generation of BM chimeras, immunoblots analysis, determination of soluble Lts in culture supernatants, and statistical analysis used in this study can be found in SI Materials and Methods.


