

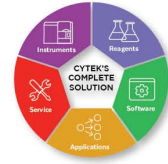


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Src Homology 2 Domain-Containing Protein Tyrosine Phosphatase Substrate 1 Regulates the Migration of Langerhans Cells from the Epidermis to Draining Lymph Nodes

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Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 (SHPS-1) is a member of the signal regulatory protein family in which the extracellular region interacts with its ligand, CD47. Recent studies have demonstrated that SHPS-1 plays an important role in cell migration and cell adhesion. We demonstrate in this study, using immunohistochemical and flow cytometric analyses, that murine Langerhans cells (LCs) express SHPS-1. Treatment of mice ears with 2,4-dinitro-1-fluorobenzene significantly reduced the number of epidermal LCs, and that reduction could be reversed by pretreatment with mAb to SHPS-1 or the CD47-Fc fusion protein. Treatment with the SHPS-1 mAb in vivo reduced the number of FITC-bearing cells in the lesional lymph nodes after the application of FITC to the skin. The SHPS-1 mAb inhibited the in vivo TNF- α -induced migration of LCs. The emigration of dendritic cells expressing I-A^{b+} from skin explants to the medium was also reduced by the SHPS-1 mAb. We further demonstrate that the chemotaxis of a murine dendritic cell line, XS52, by macrophage inflammatory protein-3 β was significantly inhibited by treatment with the SHPS-1 mAb or CD47-Fc recombinant protein. Finally, we show that migration of LCs was attenuated in mutant mice that lack the intracellular domain of SHPS-1. These observations show that the ligation of SHPS-1 with the SHPS-1 mAb or with CD47-Fc abrogates the migration of LCs in vivo and in vitro, which suggests that the SHPS-1-CD47 interaction may negatively regulate LC migration. *The Journal of Immunology*, 2004, 172: 4091–4099.

Dendritic cells (DCs)² are professional APCs that play a crucial role in the induction of immune responses to pathogens. Langerhans cells (LCs) are bone marrow-derived immature DCs that reside in the epidermis. When LCs encounter exogenous Ags, including haptens and microorganisms, they capture and process them to generate MHC/peptide complexes on their surface. LCs migrate from the epidermis to draining lymphoid tissues to initiate naive T cells and present the MHC/peptide complexes to T cells with the necessary adhesion molecules and costimulatory signals (1–7). During this migration, LCs mature from a dormant into an activated functional state. The transition from the dormant epidermal cell type to the mature lym-

phoid DC is characterized by an increase in the expression of cell surface molecules, such as MHC class II, CD80, CD86, and macrophage-derived chemokines, and a decrease in E-cadherin (7–9).

Recently, it has been shown that the migration of LC is triggered via a variety of stimuli, e.g., hapten application, injection of TNF- α , UV irradiation, or the transfer of the skin from in vivo to in vitro culture medium (3, 5, 7, 10, 11). It appears that LC migration is up-regulated by various factors, including IL-1 β , IL-16, IL-18, TNF- α , osteopontin, integrin α_6 , CD40, and PGE₂, whereas IL-10 and PGD₂ suppress LC migration (10, 12–18). Matrix metalloproteinases 9 and 2 have been shown to be necessary for the migration of LCs and dermal DCs (19). ICAM-1 deficiency in the lymphatic endothelium results in the reduced migration of LCs into draining lymph nodes (20). Recent studies have revealed that LCs are recruited into the epidermis by chemokines such as macrophage inflammatory protein-3 α (MIP-3 α /CCL20), the receptor for which (CCR6) is expressed on immature LCs. LC migration from epidermis to lymphoid tissue, in turn, requires CCR7 ligands, including MIP-3 β /CCL19 or a secondary lymphoid chemokine/CCL21 (21, 22).

One signal regulatory protein, Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 (SHPS-1) (23), is also known as signal regulatory protein α 1 (24), BIT (brain Ig-like molecule with tyrosine-based activation motifs) (25), P84 (26), macrophage fusion receptor (27), and MyD-1 (28). In mice, SHPS-1 is expressed in myeloid cells, including monocytes, macrophages, and DCs, but not in lymphocytes (29). SHPS-1 is a transmembrane glycoprotein whose extracellular domain comprises three Ig-like domains with multiple N-linked glycosylation sites, whereas the cytoplasmic domain of SHPS-1 contains four

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² Abbreviations used in this paper: DC, dendritic cell; CHS, contact hypersensitivity; DNFB, 2,4-dinitro-1-fluorobenzene; LC, Langerhans cell; MIP, macrophage inflammatory protein; SHP-1, -2, Src homology 2 domain-containing protein tyrosine phosphatase-1, -2; SHPS-1, Src homology 2 domain-containing protein tyrosine phosphatase substrate 1.

tyrosine residues that form two immunoreceptor tyrosine-based inhibitory motifs (30). SHPS-1 was initially discovered as a tyrosine-phosphorylated transmembrane protein that binds Src homology 2 domain-containing protein tyrosine phosphatases (SHP-1 or SHP-2). IAP/CD47, an integrin-associated protein, was identified as an extracellular ligand for SHPS-1 (31). The CD47-SHPS-1 system has been shown to regulate a novel cell-cell communication system important in immunology and hematology. CD47 on RBC binds to SHPS-1 on macrophages, thereby inhibiting macrophage activation and phagocytosis (32, 33). It seems that the interaction of SHPS-1 with CD47 negatively regulates cellular responsiveness during T cell activation and during the induction of Ag-specific CTL by DCs and monocytes (28, 34). CD47-SHPS-1 binding prevents the phenotypic and functional maturation of immature DCs and suppresses IL-12 production by mature DCs (35). That binding also inhibits the IL-12 responsiveness of T cells, which is caused by decreased IL-12R expression on T cells (35). CD47 ligation of umbilical cord blood mononuclear cells promotes the development of hyporesponsive T cells and induces T cell anergy (36).

It has been demonstrated that SHPS-1 plays an important role in cell movement. The ligation of the extracellular domain of SHPS-1 with an Ab or with the CD47-Fc fusion protein suppresses the migration of CHO cells and melanoma cells (37) or neutrophils (38). The migration of cultured fibroblasts from gene-manipulated mice that lack the intracellular domain of SHPS-1 is markedly reduced (39). It is believed that SHPS-1 recruits phosphatases, SHP-1 and SHP-2, which have been shown to play important roles in cell adhesion and motility (39–41). The SHPS-1-CD47 interaction may contribute to the recruitment of B lymphocytes via endothelial cells under steady state conditions (42). Thus, the interaction between SHPS-1 and CD47 is related to the migration of myeloid cells.

In this study we demonstrate that LC/DC migration in response to haptens, TNF- α , or a chemokine is significantly inhibited by the ligation of SHPS-1 with an anti-SHPS-1 mAb or the CD47-Fc recombinant protein. We further show that LC migration is impaired in SHPS-1 mutant mice (39).

Materials and Methods

Mice

The generation of mutant mice that lack most of the cytoplasmic region of SHPS-1 has been previously described (39). Mice were bred and maintained in the Institute of Experimental Animal Research of Gunma University under specific pathogen-free conditions. The mice were backcrossed onto the C57BL/6 background over five generations. Genotyping of the mice was performed by PCR analysis as previously reported (39). Mice were handled in accordance with the animal care guidelines of Gunma University. Female C57BL/6 mice (6–8 wk old) were purchased from Charles River Japan (Tokyo, Japan). All animals were maintained in microisolator cages and exposed to a 12-h light/12-h dark cycle, with standard feed and water ad libitum. All animal experiments were conducted according to the Guidelines for Animal Experimentation at Kobe University Graduate School of Medicine.

Antibodies

Ascites were collected from BALB/c *nu/nu* mice that had been injected i.p. with the hybridoma-producing rat anti-mouse SHPS-1 mAb (p84) (43). The p84 Ab was purified from the ascites using a protein A column. PE-conjugated mouse anti-mouse I-A^b mAb (AF6-120.1) and FITC-conjugated hamster anti-mouse CD11c mAb were purchased from BD Bioscience (Tokyo, Japan). FITC-conjugated goat anti-rat IgG F(ab')₂ and rat IgG were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO), respectively. Rat anti-mouse CD205 (DEC205) mAb was purchased from Serotec (Oxford, U.K.). CD47-Fc was generated as previously described (37). Briefly, CHO-Ras cells were transfected with pTracerCMV-hCD47-Fc. The CD47-Fc fusion protein was then purified from the culture supernatant by column chromatography on

Protein G HP (Amersham Pharmacia Biotech, Uppsala, Sweden). The human IgG-Fc fragment was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cells

The XS52 line, a long term DC line established from murine newborn epidermis (44), was provided by Dr. A. Takashima (University of Texas, Dallas, TX). XS52 cells were cultured in medium composed of complete RPMI (RPMI 1640 supplemented with heat-inactivated 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 100 μ M nonessential amino acids, 1 mM sodium pyruvate, 5×10^{-5} M 2-ME, and 1% penicillin/streptomycin/amphotericin B) supplemented with 2 ng/ml murine rGM-CSF (Strathmann Biotech, Hamburg, Germany) and culture supernatant (10%, v/v) from the NS47 stromal cell line (also provided by Dr. A. Takashima). To induce the maturation of XS52 cells, they were cultured in complete RPMI supplemented with 10 ng/ml murine rGM-CSF and 10 ng/ml murine rIL-4 (RERIAtech, Braunschweig, Germany). After 6 days, XS52 cells were cultured for an additional 3 days in the presence of rGM-CSF and rIL-4.

Preparation of murine epidermal sheets and immunofluorescence analysis

Ears were split into dorsal and ventral halves with the aid of forceps. 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 fixed in acetone for 3–5 min at –20°C. After fixation, the sheets were washed in PBS and then incubated at room temperature for 30 min with PE-conjugated mouse anti-mouse I-A^b mAb or FITC-conjugated hamster anti-mouse CD11c mAb, each diluted 1/100 in 5% BSA/PBS, and washed with PBS. The epidermal sheets, which were treated with rat anti-mouse-SHPS-1 mAb (1/100) or rat anti-mouse CD205 mAb (1/100), were followed by incubation for 30 min with FITC-conjugated goat anti-rat IgG F(ab')₂, diluted 1/100 in 5% BSA/PBS. Finally, the sheets were washed with PBS and mounted on microscope slides in PermaFluor (Shandon, Pittsburgh, PA). The samples were analyzed using a Fluoview confocal laser scanning microscope (Olympus, Nagano, Japan). The number of DCs seen in epidermis was from at least 10 fields/sample for each experimental condition.

Epidermal cell suspensions and analysis by flow cytometry

Dorsal and ventral ear halves were incubated in 1% trypsin (Life Technologies, Paisley, U.K.) in PBS for 60 min at 37°C. The epidermis was gently separated from the dermis using forceps. The epidermal sheets were then shaken vigorously in PBS. After the solution became cloudy, a single-cell suspension was prepared through a 30- μ m pore size cell strainer (NBC, Tokyo, Japan) and then resuspended in RPMI 1640 containing 10% FBS. After washing in RPMI 1640, the suspensions (5×10^5 /ml) were incubated on ice for 30 min with PE-conjugated mouse anti-mouse I-A^b mAb and rat anti-mouse SHPS-1 mAb, each diluted 1/100 in RPMI 1640, and were then washed with RPMI 1640. The suspensions were further incubated for 30 min with FITC-conjugated goat anti-rat IgG F(ab')₂ (1/100). After a final washing with RPMI 1640, the samples were analyzed using a FACSCalibur flow cytometer and CellQuest (BD Biosciences, San Jose, CA).

2,4-Dinitro-1-fluorobenzene (DNFB) sensitization and in vivo rat anti-mouse SHPS-1 mAb treatment

Mice were painted with 10 μ l of 0.5% DNFB solubilized in acetone/olive oil (4/1) on both the dorsal and ventral ear halves. Twenty-four hours later, the ears were collected for staining of LCs with a panel of Abs as described above. To investigate the effects of in vivo anti-mouse SHPS-1 mAb treatment, rat anti-mouse SHPS-1 mAb (p84) or control rat IgG was injected intradermally at a dose of 60 μ g/mouse 24 h before the DNFB application.

FITC-bearing LC migration assay

FITC (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in acetone/dibutylphthalate (1/1) before application. Mice were painted on the dorsal ear half with 25 μ l of 3% FITC solution. Rat anti-mouse SHPS-1 mAb and rat IgG were injected intradermally at a dose of 60 μ g/ear before the FITC application. Twenty-four hours after FITC painting, draining auricular lymph nodes were collected, placed in RPMI 1640, and teased into single-cell suspensions before total cell counts were performed and viability was checked using 0.2% Trypan Blue (Sigma-Aldrich). Single-cell suspensions were incubated on ice for 30 min with PE-conjugated mouse anti-mouse I-A^b mAb, diluted 1/100 in RPMI 1640. After a final washing with RPMI 1640, the samples were analyzed using a FACSCalibur flow cytometer and CellQuest.

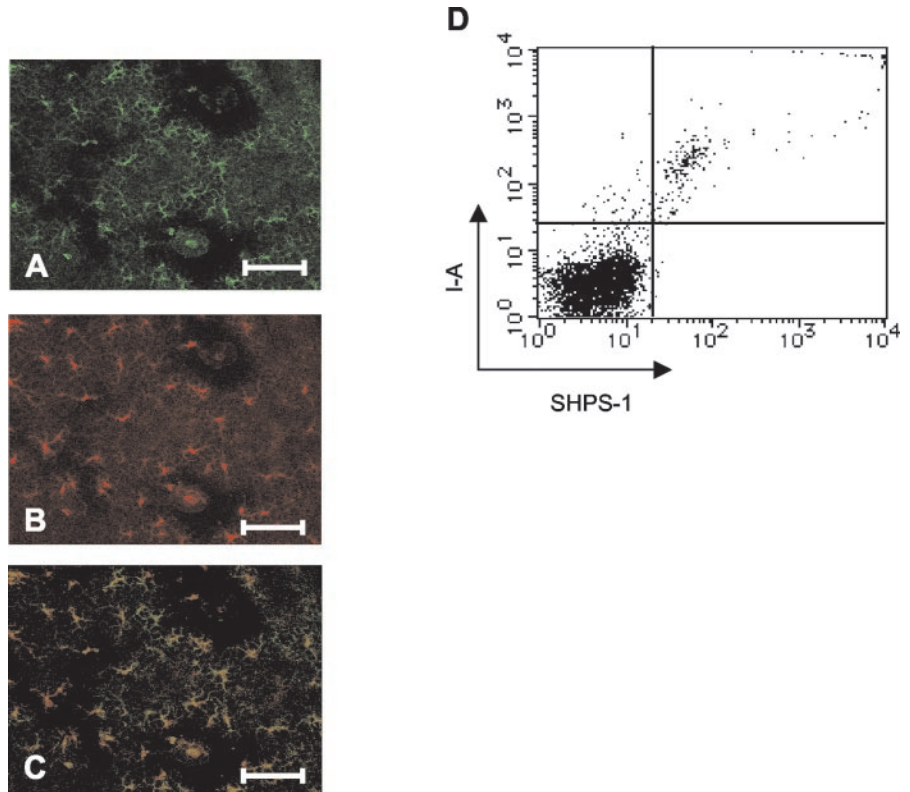


FIGURE 1. SHPS-1 is expressed on murine epidermal LCs. Green fluorescence indicates SHPS-1 (A), and red fluorescence shows I-A^b (B). A merged image of A and B is shown in C. Bar = 50 μ m. D, FACS analysis of an epidermal cell suspension, showing that the majority of I-A^b-positive cells express SHPS-1.

Skin organ culture

Ears cut off of mice were rinsed in 70% ethanol for 10 s, then split into dorsal and ventral halves. The ventral halves were cultured in 24-well tissue culture plates in 1.5 ml of culture medium for 48 h at 37°C. At least six explants were cultured for each experimental condition. The cells that had emigrated into the culture medium during this time were counted and further evaluated phenotypically using a FACSCalibur flow cytometer and Cell Quest. In some experiments epidermis was separated from dermis by incubation of dorsal halves of the ears in dispase (1 U/ml; Roche, Mannheim, Germany) for 30 min before the onset of culture (19).

TNF- α -induced LC migration assay

Groups of mice ($n = 5$) received 30- μ l intradermal injections of mouse rTNF- α (Sigma-Aldrich; 50 ng/ear) or an equivalent volume of carrier protein (0.1% BSA) into ear pinnae with 30-gauge stainless steel needles. Rat anti-mouse SHPS-1 mAb or control rat IgG was injected intradermally at a dose of 60 μ g/ear simultaneously. The ears were collected for staining of LCs 1 h after the injection.

Chemotaxis

Chemotaxicells (5- μ m pore size; Kurabo, Osaka, Japan) were pretreated with 5 μ g/ml laminin (Sigma-Aldrich) for 40 min at room temperature. XS52 cells (5×10^4) were then added to the upper chamber of the chemotaxicells, and 50 ng/ml murine rMIP-3 β (Genzyme/Techne, Minneapolis, MN) was added to the lower chamber of the chemotaxicells. After incubation for 4 h at 37°C, the membranes of the chemotaxicells were removed, fixed with methanol, and stained with Giemsa. The number of cells that transmigrated toward the lower chambers was counted under a microscope.

Statistical analysis

The statistical significance of differences between the means was determined using Student's *t* test. A difference was considered statistically significant at $p < 0.05$. Each experiment was performed at least three times.

Results

SHPS-1 is expressed on murine LC in situ

To investigate the expression of SHPS-1 on murine LCs, we performed immunofluorescent analysis using murine epidermal

sheets. Almost all cells bearing I-A^b reacted with anti-SHPS-1 mAb and showed a dendritic phenotype (Fig. 1, A–C). FACS analysis of the epidermal cells revealed similar results, showing that the majority of cells positive for I-A^b expressed SHPS-1, whereas cells without I-A^b were negative for SHPS-1 (Fig. 1D).

In vivo treatment with anti-mouse SHPS-1 mAb or CD47-Fc fusion protein prevents a decrease in LC density in epidermis after epicutaneous hapten application

To investigate the effect of an anti-mouse SHPS-1 mAb on the migration of epidermal LCs, DNFB was epicutaneously applied 24 h after treatment with anti-SHPS-1 mAb. Consequently, the population of I-A^b⁺ cells in the epidermis was decreased in mice treated with rat IgG+DNFB (Fig. 2A) compared with that in mice without DNFB treatment (Fig. 2C) (20), whereas the reduction of the I-A^b⁺ cell population in anti-SHPS-1 mAb plus DNFB treatment group was significantly inhibited (Fig. 2, B and D). The reduction of the I-A^b⁺ cell population elicited by DNFB was inhibited when CD47-Fc was injected at the site of DNFB application (Fig. 2E). The DNFB-triggered reduction of CD11c⁺ and CD205⁺ cell populations was inhibited by treatment with anti-SHPS-1 mAb (Fig. 2F) or CD47-Fc (Fig. 2G), respectively.

Reduction of I-A^b⁺ or CD11c⁺ cells on epidermal sheets induced by TNF- α is inhibited by anti-mouse-SHPS-1 mAb

It has been previously reported that TNF- α can decrease epidermal LC numbers when injected intradermally (45). Therefore, we next investigated whether *in vivo* anti-SHPS-1 mAb treatment attenuates the TNF- α -induced emigration of LC from the epidermis. Anti-SHPS-1 mAb (60 μ g/ear) and mouse rTNF- α (50 ng/ear) were simultaneously injected intradermally in a 30- μ l volume. After 1 h, the ears were collected for staining of LCs with PE-conjugated mouse anti-mouse I-A^b mAb or FITC-conjugated hamster anti-mouse CD11c mAb as previously reported (45). Intradermal injection of rTNF- α caused a reduction in the density of I-A^b⁺

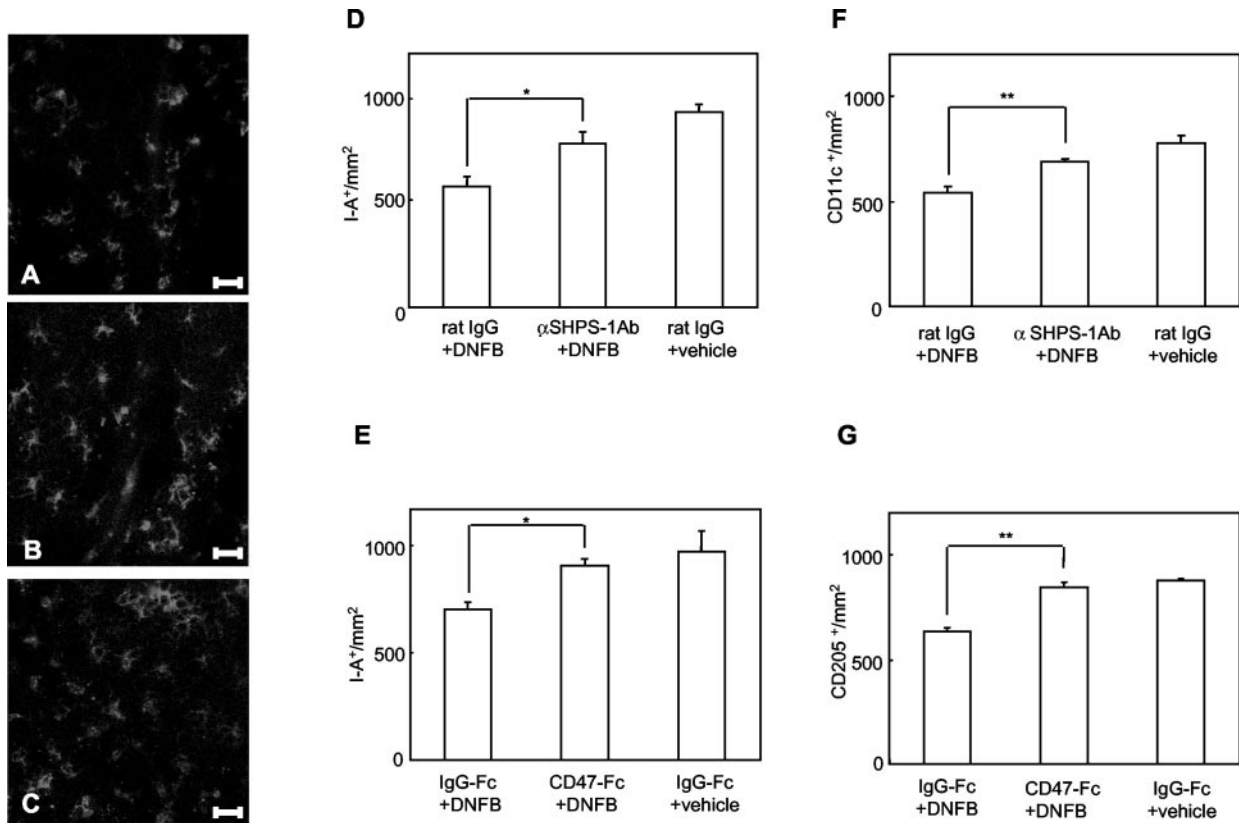


FIGURE 2. The effect of anti-mouse SHPS-1 mAb or CD47-Fc treatment on the number of LCs in the epidermis after hapten application. Rat anti-mouse SHPS-1 mAb (*B*) or rat IgG (*A* and *C*) was intradermally injected before DNFB application. The ears of the mice were then painted with 10 μ l of 0.5% DNFB solution (*A* and *B*) or vehicle (*C*). Epidermal samples were collected and stained with PE-conjugated anti-mouse I-A^b mAb 24 h later. Bars = 20 μ m. *D* and *F*, The density of I-A^b+ or CD11c⁺ cells in the epidermis is decreased in the rat IgG plus DNFB treatment group, whereas the anti-SHPS-1 mAb plus DNFB treatment group showed a lesser decrease in LC numbers. **, $p < 0.01$; *, $p < 0.05$; $n = 4$. Error bars indicate SDs. *E* and *G*, Ears of mice were injected intradermally with 30 μ g of CD47-Fc fusion protein instead of anti-SHPS-1 mAb, and then DNFB was applied to the ears. The density of I-A^b+ or CD205⁺ cells in the epidermis is decreased in the control human IgG-Fc treatment group, but not in the CD47-Fc treatment group (**, $p < 0.01$; *, $p < 0.05$; $n = 4$). Error bars indicate SDs.

epidermal LCs (data not shown). The combined injection of rat IgG+rTNF- α caused a reduction in the density of I-A^b+ epidermal LCs of 27% compared with rat IgG (Fig. 3, *A* and *D*). In contrast, the combined injection of anti-SHPS-1 mAb plus rTNF- α led to a significant attenuation of the TNF- α -induced emigration (Fig. 3, *B* and *D*). TNF- α -induced reduction of the CD11c⁺ cell population in epidermis was similarly attenuated by the combined injection of anti-SHPS-1 mAb and rTNF- α (Fig. 3*E*).

Reduction of hapten-bearing cells in the draining lymph nodes by *in vivo* anti-mouse SHPS-1 mAb treatment

To induce the migration of epidermal LC to draining lymph nodes, mice were painted with the hapten, FITC. Pretreatment with anti-SHPS-1 mAb decreased the number of FITC⁺/I-A^b+ cells migrating into the draining lymph nodes 24 h after FITC painting compared with mice pretreated with rat IgG as a control (Fig. 4*A*). In each of four separate experiments, there was a statistically significant decrease in FITC⁺/I-A^b+ cells in the draining lymph nodes in the anti-SHPS-1 mAb- plus FITC-treated group compared with the rat IgG- plus FITC-treated group (Fig. 4*B*).

Emigration of DCs and LCs from murine skin explants to culture medium is inhibited by anti-mouse SHPS-1 mAb

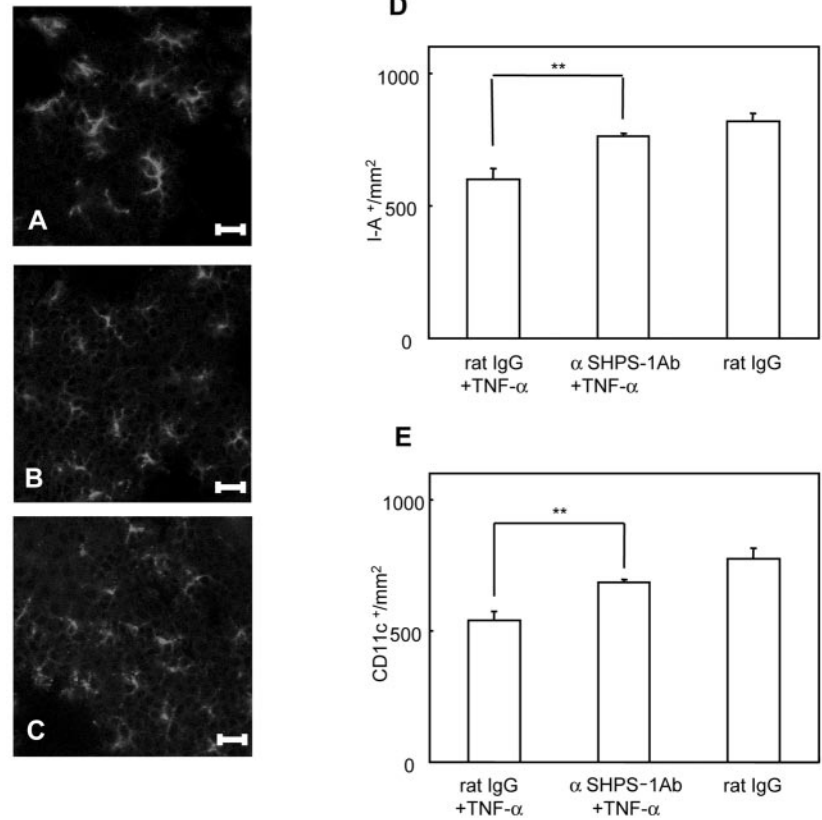
Larsen et al. (11) have established and refined a skin explant culture system that is a useful tool to further define the migratory mechanisms of cutaneous DCs. To investigate the influence of

anti-SHPS-1 mAb on the migration of cutaneous DC, we cultured murine whole skin explants with graded concentrations of anti-SHPS-1 mAb (0.5–50 μ g/ml). DCs emigrate spontaneously from murine whole skin explants into the culture medium over a period of 1–3 days. Between 2000 and 8000 DCs can be retrieved from one dorsal ear-half after 48 h. We confirmed that the phenotype of the retrieved cells was 80% positive to I-A^b by FACS analysis (not shown). When anti-SHPS-1 mAb was added to the culture medium, a dose-dependent reduction in the number of DCs retrieved from the culture medium was observed (Fig. 5*A*). The effects of 5–50 μ g/ml anti-SHPS-1 mAb were significant compared with spontaneous emigration in the absence of Ab or emigration in the presence of rat IgG (5 μ g/ml). Ratzinger et al. (19) have established a skin culture system by using epidermal sheets to investigate the emigration of epidermal DCs (LCs). Using this technique we examined the influence of anti-SHPS-1 mAb on the migration of LCs from epidermis and found that the number of LCs retrieved from the culture medium was significantly reduced by treatment with anti-SHPS-1 mAb (Fig. 5*B*).

Chemotaxis of IL-4-treated XS52 cells is attenuated by anti-mouse SHPS-1 mAb and CD47-Fc

We confirmed that XS52 cells express SHPS-1 during routine culture and after being induced to mature by IL-4 (data not shown). To obtain mature XS52 cells, they were cultured in complete

FIGURE 3. The reduction of I-A^{b+} or CD11c⁺ LCs in the epidermal sheets induced by rTNF- α is inhibited by in vivo anti-mouse SHPS-1 mAbs. Anti-mouse SHPS-1 mAb or rat IgG in combination with rTNF- α was injected intradermally. After 1 h, the epidermis was stained for I-A^{b+} or CD11c⁺, and LCs were counted using a microscope. *D*, The combined injection of rat IgG and rTNF- α caused a reduction in the I-A^{b+} LC density of 27%. Data for the combined injection of anti-mouse SHPS-1 mAb and rTNF- α was significant compared with the combined injection of rat IgG and rTNF- α ($p < 0.01$; $n = 4$). Error bars indicate SDs. Representative images of the LC population in the epidermis are shown: *A*, the rat IgG plus TNF- α treatment group; *B*, the anti-SHPS-1 mAb plus TNF- α treatment group; *C*, the rat IgG without TNF- α treatment group. Bars = 20 μm . *E*, The combined injection of anti-SHPS-1 mAb and rTNF- α attenuated the rat IgG- plus rTNF- α -induced reduction of the CD11c⁺ cell population in epidermis ($p < 0.01$; $n = 4$).



RPMI supplemented with GM-CSF and IL-4 for 6 days (IL-4XS6d), replacing the medium for an additional 3 days of culture (IL-4XS9d). IL-4XS9d was attracted to rMIP-3 β , as assessed by the chemotaxis assay, whereas IL-4XS6d did not migrate to rMIP-3 β (data not shown). Anti-SHPS-1 mAb and CD47-Fc significantly inhibited MIP-3 β -induced transmigration in dose-dependent manners (Fig. 6).

Migration of LCs induced by DNFB is attenuated in SHPS-1 mutant mice

Mice with a SHPS-1 mutation which lacks most of the cytoplasmic region of the protein and wild type mice were painted with 0.5% DNFB and after 24 h, the epidermis was removed for analysis of I-A^{b+} positive LCs. Epicutaneous DNFB application significantly

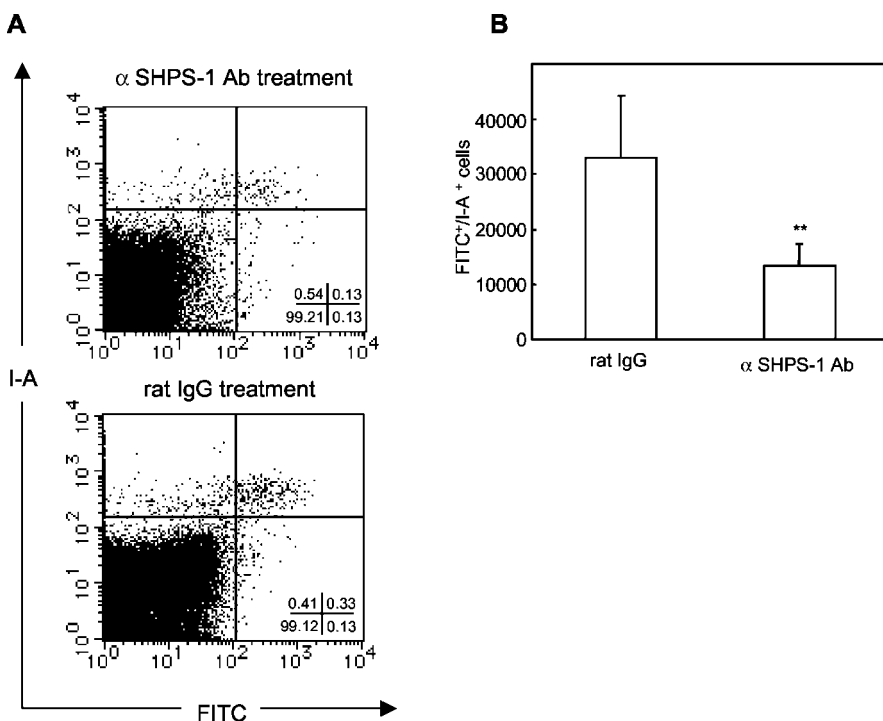


FIGURE 4. The reduction of hapten-bearing cells in the draining lymph nodes by in vivo treatment with anti-mouse-SHPS-1 mAb. *A*, Anti-mouse SHPS-1 mAb and rat IgG were injected intradermally before FITC application. Draining lymph nodes were collected 24 h after the application of 3% FITC to mice, and lymph node cells were stained for I-A^b. Representative data from FACS analyses and the percentage of FITC⁺/I-A^{b+} cells are shown. *B*, The mean number of FITC⁺/I-A^{b+} cells in the draining lymph nodes after intradermal injection of anti-mouse-SHPS-1 mAb was significantly lower compared with that after injection of rat IgG ($p < 0.01$; $n = 4$). Error bars indicate SDs.

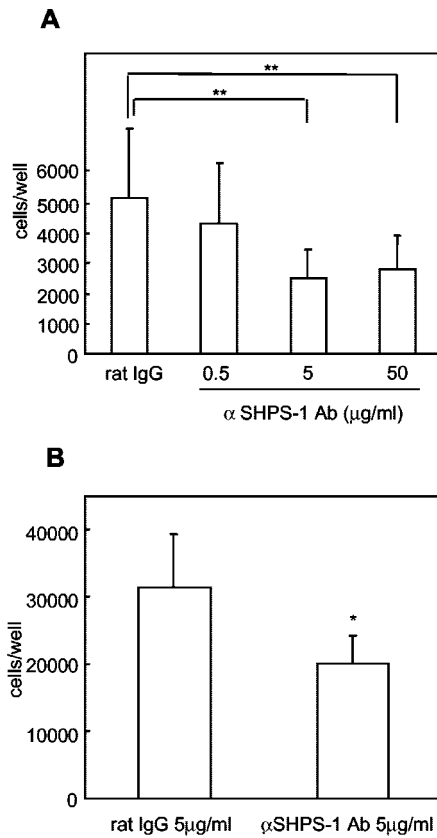


FIGURE 5. Emigration of DCs and LCs from murine skin explants to the culture medium is inhibited by anti-mouse-SHPS-1 mAb. *A*, Murine whole skin explants were placed in medium with anti-mouse SHPS-1 mAb (0.5–50 $\mu\text{g/ml}$) or rat IgG (50 $\mu\text{g/ml}$), and the emigrated DCs were counted in the medium after 48 h of culture. The inhibition was statistically significant at 5–50 $\mu\text{g/ml}$ ($p < 0.01$; $n = 6$). *B*, Epidermis was separated from dermis by dispase treatment and then cultured for 48 h. When anti-SHPS-1 mAb (5 $\mu\text{g/ml}$) was added to the culture medium, a significant reduction in the number of LCs retrieved from the culture medium was observed ($p < 0.05$; $n = 6$). Error bars indicate SDs.

decreased the epidermal LC population in wild-type mice (Fig. 7C, 7D, 7E), whereas reduction of the epidermal LC population after DNFB application in SHPS-1 mutant mice was minimal (Fig. 7A, 7B, 7E). Emigration of LCs from the epidermis of SHPS-1 mutant mice was significantly impaired as compared with that of wild-type mice (Fig. 7F).

Discussion

We show in this study that SHPS-1 is constitutively expressed on the cell surface of murine epidermal LC. FACS and immunofluorescent staining studies revealed that SHPS-1 is selectively expressed on murine LC in the epidermis, suggesting that SHPS-1 has a specific role in LC function. To address the functional role of SHPS-1 expressed on LCs, we used a well-established murine model to investigate LC migration in response to the epicutaneous application of haptens (3, 46, 47). In vivo treatment of an anti-SHPS-1 mAb or CD47-Fc fusion protein before epicutaneous DNFB application inhibited the migration of LCs from the epidermis, and pretreatment with anti-SHPS-1 mAb decreased the hapten-bearing LCs in the draining lymph nodes after FITC painting on the skin. This inhibitory effect of the SHPS-1-binding ligands on the reduction of the LC population after hapten application does not seem to be due to the enhancement of survival of LCs, because anti-SHPS-1 mAb did not affect the survival of XS52 cells and

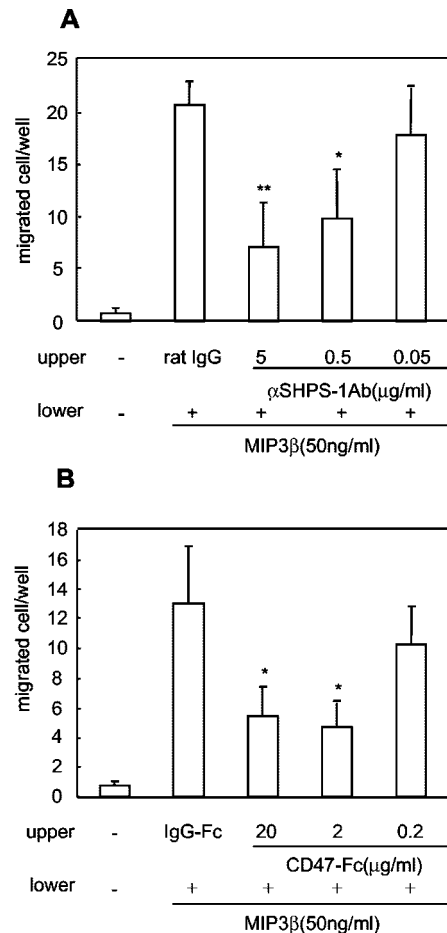


FIGURE 6. The chemotaxis of IL-4-treated XS52 cells is attenuated by anti-mouse SHPS-1 mAb. XS52 cells were cultured in complete RPMI supplemented with 10 ng/ml GM-CSF and 10 ng/ml IL-4 for 9 days, and chemotaxis for MIP-3 β was assessed. A dose-dependent reduction in the number of migrated IL-4-XS52 cells was observed when anti-mouse SHPS-1 mAb (*A*) or CD47-Fc (*B*) was added to the culture medium. *A*, Data for 0.5–5 $\mu\text{g/ml}$ were significant when compared with chemotaxis in the addition of rat IgG (5 $\mu\text{g/ml}$; **, $p < 0.01$; *, $p < 0.05$; $n = 4$). *B*, Data for 2–20 $\mu\text{g/ml}$ were significant compared with chemotaxis after addition of human IgG-Fc (20 $\mu\text{g/ml}$; $p < 0.05$; $n = 4$). Error bars indicate SDs.

LCs migrating from the skin explants (not shown). The inhibitory effect of anti-SHPS-1 mAb on LC migration was also confirmed using a skin explant culture system (19, 47). These observations indicate that SHPS-1 plays a significant role in the migration of LCs from the epidermis to draining lymph nodes after epicutaneous hapten application in vivo. As it has been shown that TNF- α is one of the critical factors that mediate the migration of LCs during hapten application on the skin (10), we next studied whether SHPS-1 is involved in the TNF- α -driven LC migration. Intradermal injection of TNF- α induced LC migration from the epidermis as previously reported (10), whereas pretreatment with an anti-SHPS-1 mAb significantly inhibited LC migration. Thus, SHPS-1 is involved in LC migration induced by TNF- α in vivo.

Mobilization of LCs from epidermis to draining lymph nodes after epicutaneous hapten application requires several steps, including the detachment of LCs from the keratinocytes, invasion from epidermis into dermis through the basement membrane zone, and migration into draining lymph vessels and finally into the draining lymph nodes. During this migration, CCR7 expression on the cell surface of LCs appears to be an essential event, because its ligands (MIP-3 β /CCL19 and secondary lymphoid chemokine/

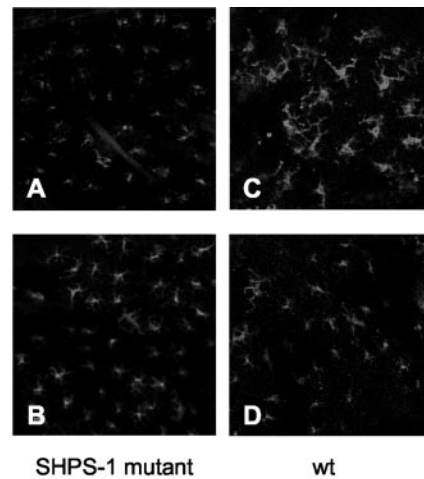
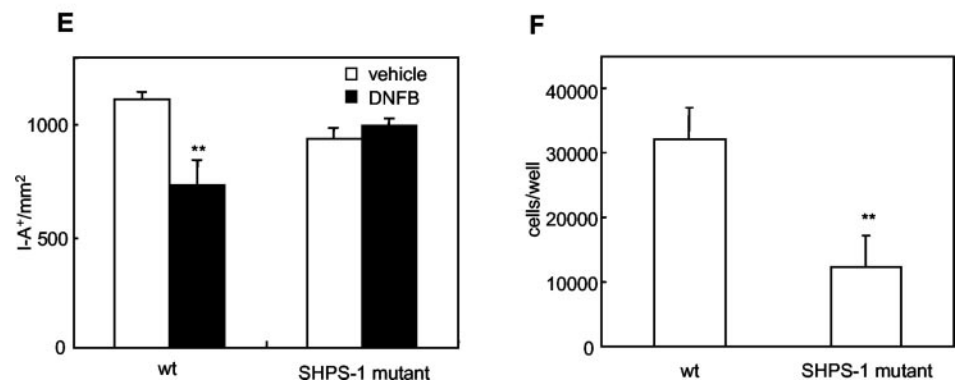


FIGURE 7. The reduction of I-A^{b+} cells in epidermal sheets by DNFB painting is attenuated in SHPS-1 mutant mice. SHPS-1 mutant (A and B) and wild-type (C and D) mice were epicutaneously painted on the ears with 0.5% DNFB (A and C) or vehicle (B and D), and the epidermis was analyzed for I-A^{b+} LC 24 h later. E, DNFB application significantly decreased the epidermal LC population in wild-type mice ($p < 0.001$; $n = 4$). In contrast, the reduction of the epidermal LC population after DNFB application in SHPS-1 mutant mice was not significant. F, Epidermis was separated from dermis using dispase. The number of LCs emigrated from the epidermal explant of SHPS-1 mutant mice was significantly reduced compared with that of wild-type mice.



CCL21) are required for LCs to migrate into lymph vessels and draining lymph nodes (21, 22). XS52 is a long term cell line established from newborn epidermis. The cells are similar to LC that are freshly obtained from the skin in terms of tissue derivation, phenotype, morphology, and Ag-presenting profile. It has been reported that IL-4 induces the phenotypic maturation of XS52 cells in a time-dependent manner (48). MIP-3 β is a potent chemoattractant for mature DCs that express MHC class II^{high} B7-2^{high} (21). We therefore investigated whether the ligation of SHPS-1 with an mAb and/or with the CD47-Fc fusion protein influences the MIP-3 β -induced migration of mature XS52 cells, which represent immature skin DCs resembling LC (44). When XS52 cells were induced to mature by the addition of IL-4 to the culture medium (48), their transmigration into the lower chamber was observed in the presence of MIP-3 β . This transmigration was significantly suppressed by the addition of an anti-SHPS-1 mAb and a synthetic peptide, CD47-Fc. Interestingly, immature XS52 cells did not migrate into the lower chamber even if MIP-3 β was added (not shown). Therefore, it is possible that CCR7 is expressed on the cell surface of mature XS52 cells stimulated with IL-4. The involvement of SHPS-1 in the cell motility induced by chemokines has not been previously reported. Whether SHPS-1 is involved in the cell motility induced by other chemokines has yet to be determined.

Evidence has gradually accumulated showing that SHPS-1 plays an important role in regulating the mechanisms of cell motility. Overexpression of SHPS-1 up-regulates cell motility and adhesion to the extracellular matrix (37), whereas cells that lack the intracellular portion of SHPS-1 move less than wild-type cells. Stimulation with growth factors and ligation of integrins with extracellular matrix induce tyrosine phosphorylation of the intracellular domain of SHPS-1, and thereby SHP-2 is recruited to form an

SHPS-1-SHP-2 complex (37). It has been shown that the motility of cells with a dominant negative form of SHP-2 is significantly decreased, indicating that recruited SHP-2 is one of the important factors that regulate cell motility (49). IL-1 β and TNF- α , which are known to induce LC migration, have been shown to induce the tyrosine phosphorylation of SHPS-1 and the consequent recruitment of SHP-2 (43). Recently, Motegi et al. (37) reported the interesting observation that engagement of SHPS-1 with an anti-SHPS-1 mAb or the CD47-Fc fusion protein leads to dephosphorylation of SHPS-1, which is associated with a decrease in cell motility. Liu et al. (38) have recently reported a similar finding that anti-SHPS-1 mAb inhibits the transmigration of neutrophils. Thus, our observations that an anti-SHPS-1 mAb or CD47-Fc inhibits the migration of LC and XS52 cells are compatible with previous findings that the ligation of SHPS-1 with its ligand or with an Ab down-regulates cell motility. We further observed that migration of LCs in mutant mice lacking the intracellular domain of SHPS-1 was significantly decreased, indicating that the intracellular domain of the SHPS-1 molecule is important for transmitting signals for motility of LCs. This is in accordance with the idea that phosphorylated tyrosine residues of the intracellular portion of SHPS-1 are important in inducing cell motility. Taken together, these data imply that the motility of epidermal LCs is down-regulated by the engagement of SHPS-1 with its ligand CD47.

SHPS-1 has an inhibitory role in the immune response when it interacts with CD47. It has been shown that ligation of SHPS-1 on DC with a mAb or CD47-Fc down-regulates IL-12 production, thereby inhibiting the activation of T lymphocytes (35). In turn, the engagement of CD47 with a mAb or SHPS-1-Fc inhibits the IL-12R on T lymphocytes, leading to T cell unresponsiveness. This proves that the ligation of SHPS-1 not only down-regulates the cell

motility of LCs, but also negatively regulates the response of immune cells. It is still unclear how the SHPS-1-CD47 system works in controlling LC migration *in vivo*. CD47 is widely expressed by various cell types, including lymphocytes. The observation that mouse keratinocytes express CD47 *in situ* (X. Yu, A. Fukunaga, and T. Horikawa, unpublished observation) suggests that SHPS-1 on LCs may be engaged with its ligand CD47 on keratinocytes, and thereby LC migration is down-regulated *in situ*. In contrast, CD47 binds to thrombospondin-1, which is produced by keratinocytes and fibroblasts (50, 51), suggesting that the ligation of SHPS-1 is competitively controlled. Recently, it has been shown that a subtype of human epidermal DCs (inflammatory dendritic epidermal cells) expresses CD36, a ligand for thrombospondin-1 (52). Thus, the regulation of LC migration by the SHPS-1-CD47 system may be a complex of these factors and should be studied further.

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