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Engagement of CD47 inhibits the contact hypersensitivity response via the suppression of motility and B7 expression by Langerhans cells

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Short title

CD47 ligation inhibits contact hypersensitivity

Abbreviations

Abbreviations used in this paper: CHS, contact hypersensitivity; DC, dendritic cell; LC, Langerhans cell; MIP, macrophage inflammatory protein; PBS, phosphate-buffered saline; PE, phycoerythrin; SD, standard deviation; SHPS-1, Src homology 2 domain-containing protein tyrosine phosphatase substrate 1; SIRP, signal regulatory protein; TNF-α, tumor necrosis factor-α.

Key words

Dendritic cells, cell surface molecules, migration, skin, costimulatory molecule.

Abstract

CD47 is a membrane-associated glycoprotein that suppresses the function of immune cells. We previously reported that Langerhans cells (LCs) express Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 (SHPS-1), a ligand for CD47, which plays an important role in the regulation of their motility. In this study, we show that LCs also express CD47, and that ligation of CD47 with SHPS-1-Fc fusion protein *in vivo* diminishes the development of the contact hypersensitivity response. We further demonstrate that CD47 engagement affects immune functions of LCs. CD47 engagement *in vivo* significantly inhibits the emigration of LCs from the epidermis into draining lymph nodes following treatment with haptens and tumor necrosis factor-α. The emigration of dendritic cells from skin explants into the medium and the chemotaxis of murine XS52 dendritic cells were significantly reduced by treatment with SHPS-1-Fc or an anti-CD47 mAb. Under explant culture system, SHPS-1-Fc treatment suppressed the expression of CD80 and CD86 of LCs. These effects on LCs and contact hypersensitivity response of CD47 ligation were reversed by treatment with pertussis toxin. These results suggest that the ligation of CD47 inhibits the migration of LCs and the expression of B7 costimulatory molecules, which results in inhibition of the contact hypersensitivity response.

Introduction

Contact hypersensitivity (CHS) is an inflammatory immune response, which is induced by the application of haptens on the skin. Exposure to antigenic stimulation by haptens stimulates Langerhans cells (LCs) in the epidermis which results in their migration from the epidermis into the draining lymphoid tissues where they initiate naïve T cells by means of major histocompatibility complex-antigen complexes (Grabbe and Schwarz, 1998; Kimber et al., 2000). During this migration, LCs undergo maturational changes that involve increases in the production of chemokines and the expression of cell surface molecules, such as major histocompatibility complex class II, ICAM-1, CD80 and CD86 (B7 molecules), which play critical roles in the development of CHS (Aiba and Katz, 1990; Cumberbatch et al., 1991; Grabbe and Schwarz, 1998; Kimber et al, 2000). The migration and maturation of LCs thus appear to be essential events in the development of CHS. The migration of LC appears to be triggered by various stimuli, including hapten application, the transfer of skin from *in vivo* to *in vitro* culture medium, and the injection of cytokines such as tumor necrosis factor-α (TNF-α) and IL-1β which are believed to play essential roles in the induction of LC migration following hapten application (Macatonia et al, 1987; Cumberbatch and Kimber, 1992; Cumberbatch et al., 1997a). LC migration is upregulated by various other factors, including IL-16, IL-18, integrin α6, CD40, and prostaglandin E_2, whereas IL-10 and prostaglandin D_2 downregulate LC migration (Price et al., 1997; Wang et al., 1999; Angeli et al., 2001; Cumberbatch et al, 2001; Stoitzner et al., 2001; Jolles et al, 2002; Kabashima et al, 2003). CCR7 in coordination with its ligand CCL19 and CCL21 appears to be indispensable in mobilizing LCs from the epidermis into lymphatics in the dermis and then into draining lymph nodes (Ohl et al, 2004).

CD47, also known as integrin-associated protein, is a 50 kDa cell-surface glycoprotein,

that is ubiquitously expressed in many cell types and is functionally associated with integrins (Brown and Frazier, 2001; Oldenborg, 2004). CD47 is composed of an immunoglobulin-like extracellular domain, five putative transmembrane-spanning segments, and a short cytoplasmic tail. One of the ligands for CD47 is thrombospondin-1, which has been shown to modulate a variety of cellular events including immune responses. Thrombospondin-1 has been shown to inhibit the production of proinflammatory cytokines including TNF-α and IL-12 by human dendritic cells (DCs) (Doyden et al, 2003). Ligation of CD47 on T lymphocytes by thrombospondin-1 suppreses the proliferation of the cells, whereas its ligation by $\alpha_4\beta_1$ integrin, another ligand for thrombospondin-1, activates adhesion, chemotaxis and matrix metalloproteinase expression of T cells (Li et al, 2002). Another ligand for CD47 appears to be Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 (SHPS-1), also known as signal regulatory protein (SIRP) α1, which is a transmembrane glycoprotein with three immunoglobulin-like extracellular domains and two immunoreceptor with tyrosine-based inhibition motifs in the cytoplasmic domain (Fujioka et al, 1996; Kharitonenkov et al, 1997; Seiffert et al, 2001). The SHPS-1-CD47 system is a novel type of cell-cell communication, which bidirectionally modulates immune responses. SHPS-1 is expressed by macrophages, DCs, neutrophils and neuronal cells, whereas CD47 is ubiquitously expressed on all hematopoietic cells and other cell types (Brown and Frazier, 2001; Oldenborg, 2004). Ligation of CD47 by a mAb to CD47 or by 4N1K, a peptide derived from the CD47-binding site of thrombospondin-1, has been shown to inhibit the development of Th1 effector cells, to downregulate the expression of IL-12R by T lymphocytes, to induce T-cell *anergy*, and to reduce cytokine production and the maturation of DCs (Avice et al, 2001; Latour et al, 2001). CD47 also plays a critical role in regulating the clearance of hematopoietic cells, including red blood cells and lymphocytes

(Oldenborg et al, 2000; Blazar et al, 2001). Engagement of CD47 has been shown to suppress transendothelial and transepithelial migration of neutrophils (Cooper et al, 1995; Parkos et al, 1996). The SHPS-1-CD47 system may play a pivotal role in regulating leukocyte-epithelial interactions (Zen and Parkos, 2003). So far, limited data are available on the function of the SHPS-1-CD47 system in immune cells *in vivo*.

We show in this study that *in vivo* treatment with a CD47 ligand significantly inhibits the development of the CHS response. We further show that treatment with a CD47 ligand inhibits not only the migration of LCs from the epidermis into draining lymph nodes but also the expression of B7 costimulatory molecules, CD80 and CD86.

Materials and Methods

Mice

Female C57BL/6 mice (6-8 weeks old) were purchased from Charles River Japan (Tokyo, Japan). All animals were maintained in microisolator cages and were exposed to a 12-hours light/12-hours dark cycle, with standard feed and water ad libitum. All animal experiments were conducted according to the Guidelines for Animal Experimentation at the Kobe University Graduate School of Medicine.

Cells

XS52 cells, a long term DC line established from murine newborn epidermis (Xu et al, 1995), were provided by Dr. A. Takashima (University of Texas, Dallas, TX). XS52 cells were cultured in medium composed of complete RPMI (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 100 μM nonessential amino acids, 1 mM sodium pyruvate, $5x10^{-5}$ M 2-mercaptoethanol (Nacalai Tesque, Tokyo, Japan), and 1% penicillin/streptomycin/amphotericin B (Bio-Whittaker, Inc., Walkersville, MD)) supplemented with 2 ng/ml murine rGM-CSF (Strathmann Biotec, Hamburg, Germany) and culture supernatant (10%, v/v) from NS47 stromal cells (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 (RELIATech, Braunschweig, Germany) (Yamada and Katz, 1999). After 6 days, XS52 cells were cultured for an additional 3 days in the presence of rGM-CSF and rIL-4. A human melanoma cell line, G361, was maintained in our laboratory, and cultured in MEM supplemented with 10% heat-inactivated fetal bovine serum (Oka et al., 1996).

Antibodies and reagents

A rat mAb to mouse CD47 (MIAP 301) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phycoerythrin (PE)- or FITC-conjugated mouse anti-mouse I-A^b mAb (AF6-120.1) was purchased from BD Bioscience (Tokyo, Japan). Rabbit polyclonal antibody raised against mouse Langerin peptide (ab22111) was from Abcam (Cambridge, UK). 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. PE-conjugated rat anti-mouse CD80 mAb and PE-conjugated rat anti-mouse CD86 mAb were from Immunotech (Marseille Cedex, France). PE-conjugated sheep anti-rabbit IgG was from Oxford Biotechnology (Kidlington, UK). Mouse SHPS-1-Fc, a chimera protein of mouse SHPS-1 and Fc portion of human IgG, was generated as previously described (Motegi et al., 2003). Briefly, CHO-Ras cells were transfected with pTracerCMV-mSHPS-1-Fc. The SHPS-1-Fc fusion protein was then purified from the culture supernatant by column chromatography on Protein A-Sepharose 4FF (Amersham Pharmacia Biotech, Uppsala, Sweden). The human IgG-Fc fragment was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). FITC-conjugated goat affinity purified $F(ab')_2$ fragments to human IgG were purchased from ICN Pharmaceuticals (Aurora, CA). Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA).

Flow cytometry analysis

Epidermal cell suspensions were obtained from mouse ears as previously described (Fukunaga et al., 2004). The suspensions ($5x10^5$/ml) were incubated on ice for 30 minutes with PE-conjugated mouse anti-mouse I-A^b mAb and rat anti-mouse CD47 mAb or SHPS-1-Fc, each at a 1/100

dilution in RPMI 1640, and were then washed with RPMI 1640. The suspensions were further incubated on ice for 30 minutes with FITC-conjugated goat anti-rat IgG $F(ab')_2$ or with FITC-conjugated goat anti-human IgG (whole molecule), each diluted 1/100 in RPMI 1640. The XS52 cells ($1x10^6$/ml) were incubated on ice for 30 minutes with rat IgG and rat anti-mouse CD47 mAb, each diluted 1/100 in RPMI 1640, and were then washed with RPMI 1640. The cells were further incubated on ice for 30 minutes 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). To examine if engagement of CD47 by SHPS-1-Fc inhibits the binding of CD47 mAb, XS52 cells were preincubated with SHPS-1-Fc for 30 minutes and then were incubated with the CD47 mAb, followed by incubation with FITC-conjugated goat anti-rat IgG $F(ab')_2$.

Preparation of murine epidermal sheets and immunofluorescence analysis

Epidermal sheets were obtained as previously described (Fukunaga et al., 2004). 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 hours at 37ºC. The epidermis was then separated from the dermis using forceps and was washed in phosphate-buffered saline (PBS). Epidermal sheets were fixed in cold acetone for 3-5 minutes at -20ºC. After fixation, the sheets were washed in PBS and were then incubated at room temperature for 30 minutes with FITC-conjugated mouse anti-mouse $I\text{-}A^b$ mAb and PE-conjugated rat anti-mouse CD80 mAb or PE-conjugated rat anti-mouse CD86 mAb, each diluted 1/100 in 5% BSA/PBS. The sheets were finally 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 LCs

seen in the epidermis was counted in 10 fields/sample for each experimental condition.

Contact hypersensitivity and histological examination

CHS response was assessed as previously described with a slight modification (Ando et al., 2000). Sensitization was induced on day 0 by applying 10 μl DNFB solution (0.5% in acetone/olive oil, 4/1) to the dorsal aspect of each right ear. After 5 days, 10 μl of 0.2% DNFB solution was applied to the dorsal surface of each left ear. Non-sensitized mice were evaluated in parallel to determine the skin irritant component of the DNFB challenge solution. Ear swelling was measured in a blinded fashion with a digimatic micrometer (Mitutoyo, Kawasaki, Japan) 24 hours after challenge. To investigate the effects of *in vivo* SHPS-1-Fc treatment on CHS, 60 μg SHPS-1-Fc or control human IgG-Fc in 30 μl of PBS was injected intradermally into the right dorsal ear just before sensitization. Some mice that were treated with SHPS-1-Fc were intradermally injected with 30 μl of 3.3 μg/ml pertussis toxin or 30 μl of control PBS 2 hours before the SHPS-1-Fc treatment. Each group consisted of at least five mice. Ear tissues were surgically removed from mice immediately after determination of ear swelling, fixed in 10% formalin, and then processed and stained with hematoxylin and eosin.

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 both the dorsal and ventral ear halves with 25 μl of 3% FITC solution. The SHPS-1-Fc fusion protein and human IgG-Fc were injected intradermally at a dose of 60 μg/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 and viability were checked using 0.2% Trypan Blue (Sigma-Aldrich). Single-cell suspensions were incubated on ice for 30 minutes 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. For intracellular staining of Langerin, we used the saponin technique (Ohl et al., 2004). We fixed the lymph node cells with 0.5% paraformaldehyde for 10 minutes on ice, followed by treatment with 0.1% saponin in PBS containing 3% FCS for 15 minutes at room temperature. The cells were then incubated with anti-mouse Langerin polyclonal antibody (1:100 dilution) on ice for 30 minutes, washed, and incubated with PE-conjugated anti-rabbit IgG (1:50 dilution) on ice for 30 minutes. After final washing, samples were analyzed by FACSCaliber. The numbers of migrated FITC bearing-I-A^{b+} cells in the draining lymph nodes were calculated using the following formula: the numbers of migrated FITC bearing cells = (the number of cells in the lymph node) (the percentage of FITC bearing-I-A^{b+} or FITC bearing-Langerin$^+$ cells)/100.

TNF-α-induced LC migration assay

Groups of mice (n = 5) received intradermal injections of 30 μl mouse rTNF-α (Sigma-Aldrich; 60 ng/ear) or an equivalent volume of carrier protein (0.1% BSA) into ear pinnae with 30-gauge stainless steel needles. SHPS-1-Fc or control human IgG-Fc was injected intradermally at a dose of 60 μg/ear simultaneously. The ears were collected for staining of LCs 1 hour after the injection (Cumberbatch et al., 1997b).

Epidermal skin organ culture

Epidermal skin organ culture was performed as previously described (Ratzinger et al., 2002).

Ears cutoff from mice were rinsed in 70% ethanol for 10 seconds and were then split into dorsal and ventral halves. The epidermis was separated from the dermis by incubation of the dorsal halves of the ears in dispase II (1.2 U/ml; Roche, Penzbeg, Germany) for 30 minutes before the onset of culture (Fukunaga et al., 2004). These pieces of epidermis were cultured in 24-well tissue culture plates in 1.5 ml of 10% fetal bovine serum RPMI1640 for 48 hours at 37ºC. At least six explants were cultured for each experimental condition. The cells that had emigrated into the culture medium during this time were counted. In some experiments, emigrated cells were collected and incubated at room temperature for 30 minutes with FITC-conjugated mouse anti-mouse I-A^b mAb and PE-conjugated rat anti-mouse CD80 mAb or PE-conjugated rat anti-mouse CD86 mAb, each diluted 1/100 in 5% BSA/PBS. The samples were analyzed using a FACSCalibur flow cytometer and CellQuest.

Chemotaxis

Chemotaxicells (5 μm pore size; Kurabo, Osaka, Japan) were pretreated with 5 μg/ml laminin (Sigma-Aldrich) for 40 minutes at room temperature. XS52 cells ($5x10^4$) were then added to the upper chamber of each chemotaxicell, and 50 ng/ml murine rMIP-3β (Genzyme/Techne, Minneapolis, MN) was added to the lower chamber. After incubation for 4 hours at 37°C, the membranes of the chemotaxicells were removed, fixed with methanol, and stained with Giemsa. The number of cells that had transmigrated toward the lower chamber was counted using a microscope.

Statistical analysis

The statistical significance of differences between the means was determined using the

two-tailed Student's t-test. A difference is considered statistically significant at $p < 0.05$. Each experiment was performed at least two times.

Results

Epidermal LCs in situ, XS52 cells and keratinocytes express CD47

FACS analyses of freshly isolated epidermal cells revealed that I-A^b-positive cells and I-A^b-negative cells in the epidermis are recognized by a CD47 mAb (Fig 1A). SHPS-1-Fc is a chimera protein of human Fc portion of human IgG and mouse SHPS-1, the ligand for CD47. Similar to the CD47 mAb, SHPS-1-Fc also binds to I-A^b-positive and I-A^b-negative cells (Fig 1B). The binding of SHPS-1-Fc to the plasma membrane of LC-like XS52 cells was confirmed by FACS (Fig 1C). G361, a human melanoma cell line that does not express mouse CD47, did not bind to SHPS-1-Fc (Fig 1D) or CD47 mAb (not shown). Preincubation with SHPS-1-Fc reduced the binding of the CD47 mAb to XS52 cells by a maximum of 70% (Fig 1E). Preincubation with CD47 mAb reduced the binding of SHPS-1-Fc to XS52 cells by a maximum of 90% (Fig 1F). These observations indicate that the binding of SHPS-1-Fc is specific to CD47.

Treatment with SHPS-1-Fc in vivo during the sensitization phase but not challenge phase reduces the CHS response

Because it has been shown that ligation of CD47 modulates immune functions in vitro, we examined whether treatment with SHPS-1-Fc *in vivo* affects the development of CHS. When mice were sensitized by treatment with control human IgG plus DNFB, significant ear swelling with multiple infiltrating leukocytes was observed (Fig. 2C, D) compared to unsensitized controls (Fig. 2A, D). However, treatment with SHPS-1-Fc+DNFB significantly reduced the ear swelling after challenge compared to controls treated with human IgG-Fc+DNFB (Fig. 2B, D). Treatment with SHPS-1-Fc before DNFB challenge did not affect the increment of ear swelling (SHPS-1-Fc, 227 ± 29 μm vs control human IgG-Fc, 232 ± 48 μm).

In vivo SHPS-1-Fc treatment reduces the number of hapten bearing cells in lymph nodes

Based on the observation that treatment with SHPS-1-Fc reduced the development of CHS, we hypothesized that engagement of CD47 *in vivo* would influence the motility of LC. To test that hypothesis, we first analyzed draining lymph nodes of mice, which had been treated with SHPS-1-Fc and then painted with FITC. Pretreatment with SHPS-1-Fc (60 μg/ear) decreased the number of $FITC^{+}/I\text{-}A^{b+}$ cells migrating into the draining lymph nodes 24 hours after the FITC painting compared with mice pretreated with human IgG-Fc as a control (Fig. 3A). In each of four separate experiments, there was a statistically significant decrease in $FITC^{+}/I\text{-}A^{b+}$ cells in the draining lymph nodes of the SHPS-1-Fc+FITC treated group compared with the human IgG-Fc+FITC-treated group (Fig. 3C). To further confirm the inhibitory effect of treatment with SHPS-1-Fc on the migration of LCs into the lymph nodes, we assessed the number of $FITC^{+}/Langerin^{+}$ cells in the draining lymph nodes after the application of FITC. Similar to $FITC^{+}/I\text{-}A^{b+}$ cells, we observed a significant decrease in $FITC^{+}/Langerin^{+}$ cells in the draining lymph nodes of the SHPS-1-Fc+FITC-treated group compared with the human IgG-Fc+FITC-treated group (Fig. 3B, 3D).

In vivo treatment with SHPS-1-Fc fusion protein prevents a decrease of LC density in the epidermis after epicutaneous hapten application and TNF-α treatment

To examine whether SHPS-1-Fc affects the migration of LCs from the epidermis into the dermis, the epidermis was obtained 24 hours after the epicutaneous application of DNFB following treatment with SHPS-1-Fc. Consequently, the population of $I\text{-}A^{b+}$ cells in the epidermis was decreased in mice treated with human IgG-Fc+DNFB (Fig. 4C) compared to mice without

DNFB treatment (Fig. 4A), whereas the reduction of the I-A^{b+} cell population in the SHPS-1-Fc+DNFB-treated group was significantly inhibited (Fig. 4B, 4D). Similarly, SHPS-1-Fc reduced the decrease in the number of I-A^{b+} cells in the epidermis after TNF-α treatment (Fig. 4E).

Emigration of LCs from epidermal explants to the culture medium is inhibited by SHPS-1-Fc or by a CD47 mAb

It has been shown that epidermal LCs emigrate into the culture media during skin explant culture (Ratzinger et al., 2002). To investigate the influence of ligation of CD47 on the migration of epidermal LC, we cultured murine epidermal explants with graded concentrations of SHPS-1-Fc (0.3-30 μg/ml) or a CD47 mAb (0.2-20 μg/ml). LCs emigrate spontaneously from murine epidermal explants into the culture medium over a period of 48 hours. We confirmed by FACS analysis that 80% of the retrieved cells were positive for I-A^{b} (not shown). When SHPS-1-Fc was added to the culture medium, a significant reduction in the number of LCs retrieved from the culture medium was observed at concentrations of 3-30 μg/ml (Fig. 5A). The addition of a CD47 mAb similarly reduced the number of LCs retrieved from the culture medium at concentrations of 2-20 μg/ml (Fig. 5B). It has been well documented that Gi protein is associated with CD47, and that a Gi/o inhibitor, pertussis toxin, inhibits CD47-mediated cellular response (Frazier et al., 1999). We therefore investigated whether pertussis toxin could suppress the inhibitory effect of CD47 ligation on the motility of LC. Treatment with pertussis toxin significantly reversed the inhibitory effect of SHPS-1-Fc on LC migration into the culture medium (Fig. 5C).

The chemotaxis of IL-4-treated XS52 cells is attenuated by SHPS-1-Fc and by a CD47 mAb

Because immature XS52 cells do not respond to stimulation with macrophage inflammatory protein (MIP)-3β, we used mature XS52 cells in the chemotaxis assay as previously described (Fukunaga et al., 2004). To obtain mature XS52 cells, they were cultured in complete RPMI supplemented with GM-CSF and IL-4 for 9 days (then named mature XS52 cells). Mature XS52 cells were attracted to rMIP-3β as assessed by the chemotaxis assay, whereas SHPS-1-Fc or a CD47 mAb significantly inhibited the MIP-3β-induced transmigration in dose-dependent manners (Fig. 6A, 6B). The addition of pertussis toxin significantly reversed the inhibitory effect of SHPS-1-Fc on LC transmigration (Fig. 6C). SHPS-1-Fc or CD47 mAb did not affect the survival of XS52 cells for 24 hours, as confirmed by the Trypan blue exclusion test (not shown).

Treatment with SHPS-1-Fc downregulates the expression of CD80 and CD86

As B7 costimulatory molecules, CD80 and CD86, play essential roles in the development of CHS, we next questioned whether treatment with SHPS-1-Fc would affect the expression of these costimulatory molecules. It has been shown that LCs increase expression of CD86 during explant culture (Ohl et al., 2004). To study the expression of CD86, we used the epidermal explant system in which SHPS-1-Fc inhibits the migration of LCs from the epidermal explant into the culture media. The explants were collected from the culture wells 24 hours after the incubation and were stained for I-A^b and CD86. Explant cultures increased the expression of CD86 in I-A^{b+} cells in the epidermal explants (Fig. 7A, upper left and upper right) compared with *in situ* (Fig. 7A, lower left and lower right), whereas treatment with SHPS-1-Fc inhibited the increase of CD86 expression (Fig. 7A, middle left and middle right). We assessed the CD86-positive area in each I-A^{b+} cell by computer image analysis (NIH image) and observed a statistically significant reduction of CD86 expression (Fig. 7B). Similarly CD80 expression was

reduced by the addition of SHPS-1-Fc to the culture medium during the explant culture, although the intensity of the expression was weaker than CD86 (Fig. 7C). The reduction of CD86 expression was again reversed by treatment with pertussis toxin (Fig. 7D). To further confirm the inhibitory effect of CD47 ligation on the B7 expression of LCs, we analysed the expression of B7 on the emigrated cells into the medium during skin explant culture. SHPS-1-Fc reduced the expression of CD80 (Fig. 8A) and CD86 (Fig. 8B) on the I-A-bearing emigrated cells. These results indicate that ligation of CD47 by SHPS-1-Fc reduces the expression of B7 molecules.

Pertussis toxin reverses the attenuated CHS response elicited by SHPS-1-Fc treatment

Based on the finding that pertussis toxin reverses the inhibitory effect of SHPS-1-Fc on the motility and the expression of costimulatory molecules of LCs, we examined whether pertussis toxin could block the inhibitory effect of SHPS-1-Fc on the development of CHS. We pretreated the mice with pertussis toxin 2 hours before the treatment with SHPS-1-Fc, and then sensitized the mice. This procedure significantly reversed the inhibitory effect of SHPS-1-Fc on the development of the CHS response (Fig. 9).

Discussion

Although accumulating evidence has revealed that the SHPS-1-CD47 system modulates immune systems (Cooper et al., 1995; Parkos et al., 1996; Oldenborg et al., 2000; Avice et al., 2001; Brown and Frazier, 2001; Blazar et al., 2001; Latour et al., 2001; Seiffert et al., 2001; Zen and Parkos, 2003; Oldenborg, 2004), there is limited information about the effects of ligation of these molecules on T-cell-mediated immune responses *in vivo*. Because LCs express CD47, we hypothesized that engagement of CD47 may modulate LC functions and consequently the development of CHS. We showed here that *in vivo* treatment with SHPS-1-Fc inhibits the development of the CHS response, a representative immune response in which antigen-primed T cells mediate the inflammation. As the migration of LCs from the epidermis into the draining lymph nodes is a critical event in the sensitization phase of CHS, we sought to address the possibility that CD47 engagement downregulates the migration of LC. We found that the engagement of CD47 with SHPS-1-Fc reduced the motility of LCs triggered by haptens, by TNF-α and by transferring the epidermis from *in vivo* to culture medium. LC migration into draining lymph nodes elicited by FITC painting on the skin was significantly reduced by treatment with SHPS-1-Fc. As the migration of LCs into draining lymph nodes is a key event in generating antigen-specific memory T cells and the consequently developing CHS, suppression of LC motility could be one reason why treatment with SHPS-1-Fc attenuates development of the CHS response during the sensitization phase.

We further observed that SHPS-1-Fc or a CD47 mAb significantly reduced the upregulated expression of B7 molecules on the surface of LCs during skin explant culture. It has been shown that transferring skin from *in vivo* to *in vitro* culture medium induces an upregulated expression of CD86 by LCs (Ohl et al., 2004), similar to what occurs following hapten

application *in vivo* (Nuriya et al., 1996). Thus, skin explant culture mimics the effect of hapten application on changes in epidermal LCs, that is, enhanced motility and an increased expression of costimulatory molecules. Our finding that the engagement of CD47 suppresses costimulatory molecules on LCs is in accordance with the observation that engagement with a CD47 mAb inhibits the maturation of human DCs which includes the expression of CD80 and CD86, resulting in T-cell anergy (Demeure et al., 2000; Avice et al., 2001). As the expression of B7 molecules on LCs seems to be indispensable in antigen presentation during the sensitization phase of CHS, the CHS response could be impaired by the downregulation of these two costimulatory molecules and/or the suppression of LC motility by CD47 engagement. Because it is well known that B7 molecules are expressed during the maturation of DCs including LCs, the engagement of CD47 with SHPS-1 may downregulate the maturation of LCs.

CD47 is expressed on various cell types including LCs and keratinocytes, suggesting that *in vivo* injection of SHPS-1-Fc may bind to all these cells. The engagement of CD47 on keratinocytes or LCs by SHPS-1-Fc appears to suppress the migration of LCs, because SHPS-1-Fc decreases the migration of LCs from explant cultures of epidermis where no other cells are present that could influence the motility of LCs. Further, treatment with SHPS-1-Fc or a CD47 mAb reduced the transmigration of XS52 cells stimulated with MIP-3β, which suggests that the direct engagement of CD47 on DCs inhibits their motility rather than affecting surrounding cells. This observation also suggests that chemokine-induced LC migration is modulated by CD47 engagement. The evidence that the TNF-α-driven migration of LCs is suppressed by treatment with SHPS-1-Fc suggests that SHPS-1-Fc suppresses LC migration following TNF-α signaling rather than inhibiting TNF-α production by keratinocytes. Taken together, the results imply that the inhibitory effect of *in vivo* treatment with SHPS-1-Fc on LC

motility is mediated via the direct binding of SHPS-1-Fc with CD47 on LCs. This is compatible with the observation that engagement of CD47 with a CD47 mAb or 4N1K reduces the transmigration of neutrophils (Cooper et al., 1995; Parkos et al., 1996). Because thrombospondin-1 is another ligand for CD47, and downregulates DC functions including cytokine production (Doyen et al., 2003), it is possible that CD47 ligation by thrombospondin-1 also modulates LC functions, including motility.

The inhibitory effect of treatment with SHPS-1-Fc on the induction of CHS was reversed by pretreatment with pertussis toxin. Pretreatment with pertussis toxin and subsequent treatment with SHPS-1-Fc reversed the downregulation of motility and the expression of B7 costimulatory molecules by LCs. The inhibitory effect of CD47 engagement by SHPS-1-Fc on the development of CHS and the modification of LCs is thus mediated via Gi/o protein. Although it is clear that pertussis toxin enhances the responses of CHS and delayed-type hypersensitivity (Gamble et al., 1983; Sewell et al., 1983; Roberts et al., 1985), the mechanism(s) of the pertussis toxin-induced enhancement of CHS and delayed-type hypersensitivity is not understood. In the epidermis, CD47 and SHPS-1 on different LCs may bind to each other via their dendrites or both on the same LC may interact in a cis fashion (Zen and Parkos, 2003). Furthermore, it has been shown that SHPS-1 is shed from the plasma membrane, suggesting that soluble SHPS-1 may bind to CD47 (Ohnishi et al., 2004). The engagement of CD47 with SHPS-1 may signal LCs in a Gi protein-dependent manner to downregulate their motility, their expression of costimulatory molecules, and/or consequently suppress the CHS response. We therefore assume that the enhancing effect of pertussis toxin on the sensitization of CHS is, at least in part, via inhibition of the CD47/Gi complex-mediated signaling which suppresses LC migration as well as the expression of B7 molecules.

On the other hand, in the case of human neutrophils, which express SHPS-1/SIRPα, the binding of CD47 and SHPS-1/SIRPα is required in the transmigration through epithelial and endothelial tissues (Cooper et al., 1995; Parkos et al., 1996; Liu et al., 2001). CD47-null mice have been shown to develop *E. coli* sepsis because of a delayed recruitment of neutrophils to the infected organ (Lindberg et al., 1996). Thus, CD47 seems to be a prerequisite molecule in neutrophil transmigration through epithelial and endothelial organs. Ligation of CD47 *in vivo* by exogenously injected SHPS-1-Fc may interfere with the CD47-SHPS-1 interaction and thereby affect the migration of LCs. Because keratinocytes express SHPS-1 at very low levels, SHPS-1-Fc blocks the binding of CD47 on keratinocytes and SHPS-1 on LCs. Previously, we showed that engagement of SHPS-1 on LCs attenuates the motility of LCs, suggesting that the binding of SHPS-1 to CD47 on keratinocytes downregulates LC migration (Fukunaga et al., 2004). If this is the case, the engagement of CD47 on keratinocytes with SHPS-1-Fc and the consequent reduction of the ligation of SHPS-1 on LCs might prevent the downregulation of LC migration. However, treatment with SHPS-1-Fc *in vivo* suppresses the migration of LCs. We therefore speculate that blocking the binding of SHPS-1 and CD47 may not affect the suppression of LC motility.

CD47-SHPS-1 interaction may induce a firm adhesion, resulting in the aggregation of cells (Babic et al., 2000). Because the migration of LCs requires their detachment from keratinocytes, it is possible that firm adhesion between LCs and keratinocytes may disturb the detachment and the subsequent migration of LCs. Very recently, it has been shown that CD47-expressing cells firmly adhere to other CD47-expressing cells without interacting with SHPS-1/SIRPα or with thrombospondin-1 (Rebres et al., 2005). A possible homophilic binding of CD47 molecules is proposed as a mechanism for this CD47-mediated aggregation of cells.

This firm adhesion is inhibited by pertussis toxin, suggesting that Gi/o protein-CD47-associated signaling is involved. The engagement of CD47 with 4N1K upregulates the CD47-dependent cell-cell adhesion, whereas binding with a CD47 mAb (2D3) inhibits the adhesion (Rebres et al., 2005). Whether CD47-dependent adhesion between LCs and keratinocytes is enhanced by the ligation of CD47 with SHPS-1-Fc is so far unclear, and should be further studied.

The engagement of CD47 has been shown to modulate antigen presentation *in vitro*. The engagement of CD47 by a CD47 mAb and by 4N1K inhibits the proliferation of lymphocytes in mixed lymphocyte reaction or induces anergy (Avice et al., 2001). Recently, SIRPβ2 has been found to be a new ligand for CD47. SIRPβ2 lacks an immunoreceptor with tyrosine-based inhibition motif in its sequence and is expressed by T cells. The binding of SIRPβ2 on T cells and CD47 on DCs costimulates the proliferation of T lymphocytes (Piccio et al., 2005). Treatment with SHPS-1-Fc *in vivo* may inhibit the binding of these two molecules in secondary lymphoid tissues, and thereby it may prevent the development of CHS. Thus, a possible inhibitory effect of SHPS-1-Fc on SIRPβ2-mediated antigen presentation suggests a further mechanism for SHPS-1-Fc to inhibit the development of CHS.

In conclusion, we show in this study that *in vivo* treatment with a CD47 ligand, SHPS-1-Fc, reduces the development of CHS, as well as the motility and costimulatory molecule expression of LCs. As the engagement of SHPS-1 by CD47 also inhibits LC migration, SHPS-1-CD47 mutually inhibits the functions of LCs for the development of CHS.

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Figure legends

Figure 1. CD47 is expressed on murine epidermal LCs or XS52 cells. FACS analysis of an epidermal cell suspension shows that the majority of I-A^b-positive cells (A) express CD47 and (B) bind to SHPS-1-Fc. The left panels of (A) or (B) depict anti-rat IgG-FITC or human IgG-Fc, respectively. (C) The histogram obtained by FACS analysis using SHPS-1-Fc and FITC-conjugated goat anti-human IgG indicates a significant expression of CD47 on the cell surface of XS52 cells (dashed line) as compared to human IgG-Fc (solid line). (D) G361, a human melanoma cell line that does not express mouse CD47, did not show binding of SHPS-1-Fc (dashed line) or control human IgG-Fc (solid line) on its cellular surface. (E) Preincubation with SHPS-1-Fc reduced the binding of anti-mouse CD47 mAb on XS52 cells (dotted line) compared to that without preincubation with SHPS-1-Fc (bold line). (F) Preincubation with anti-mouse CD47 mAb reduced the binding of SHPS-1-Fc on XS52 cells (dotted line) compared to that without preincubation with anti-mouse CD47 mAb (bold line).

Figure 2. Treatment with SHPS-1-Fc *in vivo* suppressed the development of CHS. (B) SHPS-1-Fc or (A, C) human IgG-Fc was injected intradermally into the right ear before application of 0.5% DNFB solution. After 5 days, 0.2% DNFB was applied on the left ear and ear swelling was evaluated 24 hours after application. (C) The infiltration of leukocytes was profound in mice of IgG-Fc + DNFB sensitization group, whereas (A) minimum or (B) reduced infiltrates were observed in IgG-Fc without sensitization or in SHPS-1-Fc + DNFB groups, respectively. (D) Ear swelling for SHPS-1-Fc treatment is significantly decreased compared with human IgG-Fc (**$p < 0.01$; n = 5). Error bars indicate standard deviatins (SDs). Bars = 100 μm.

Figure 3. The reduction of hapten-bearing cells in draining lymph nodes by *in vivo* treatment with SHPS-1-Fc. SHPS-1-Fc and human IgG-Fc were injected intradermally before FITC application. Draining lymph nodes were collected 24 hours after the application of 3% FITC, and lymph node cells were stained for I-A^b or Langerin. A total of 100,000 and 30,000 cells were analysed using FACScaliber for $FITC^+$/I-A^{b+} and $FITC^+$/$Langerin^+$ cells, respectively. Representative data from FACS analyses and the percentage of (A) $FITC^+$/I-A^{b+} cells and (B) $FITC^+$/$Langerin^+$ cells are shown. The mean numbers of (C) $FITC^+$/I-A^{b+} cells and (D) $FITC^+$/$Langerin^+$ cells in the draining lymph nodes after intradermal injection of SHPS-1-Fc plus FITC application were significantly lower compared with that after injection of human IgG-Fc plus FITC application (**$p < 0.01$; n = 4). Error bars indicate SDs.

Figure 4. The inhibitory effect of SHPS-1-Fc treatment on the reduction of I-A^{b+} LCs in the epidermal sheets induced by DNFB and rTNF-α treatment. SHPS-1-Fc or human IgG-Fc was intradermally injected into mouse ears in combination with DNFB application. After 24 hours, epidermal sheets were obtained and I-A^{b+} cells were visualized using immunofluorescence. Representative images of the LC population in the epidermis are shown: (A) human IgG-Fc without DNFB treatment group; (B) SHPS-1-Fc plus DNFB treatment group; and (C) human IgG-Fc plus DNFB treatment group. Bars = 20 μm. (C, D) Pretreatment with human IgG-Fc and DNFB application caused a 36.2% reduction in the I-A^{b+} LC density. (B, D) Pretreatment with SHPS-1-Fc and DNFB application suppressed the reduction in the I-A^{b+} LC density by 11.2%. Data for the combined treatment of SHPS-1-Fc and DNFB were significant compared with the combined treatment of human IgG-Fc and DNFB (**$p < 0.01$; n = 6). Error bars indicate SDs.

(E) rTNF-α or PBS was injected intradermally. After 1 hour, the epidermis was stained for I-A^{b+}, and LCs were counted using a microscope. The combined injection of human IgG-Fc and rTNF-α caused a 36.3% reduction in the I-A^{b+} LC density. The combined injection of SHPS-1-Fc and rTNF-α attenuated the reduction in the I-A^{b+} LC density by 17.6%. Data for the combined injection of SHPS-1-Fc and rTNF-α were significant compared with the combined injection of human IgG-Fc and rTNF-α (**$p < 0.01$; n = 6). Error bars indicate SDs.

Figure 5. The emigration of LCs is inhibited by SHPS-1-Fc or a rat anti-mouse CD47 mAb. Epidermis was separated from dermis by dispase treatment and then placed in medium with (A) SHPS-1-Fc (0.3-30 μg/ml) or human IgG-Fc (30 μg/ml) and (B) rat anti-mouse CD47 mAb (0.2-20 μg/ml) or rat IgG (20 μg/ml). Emigrated LCs were counted in the medium after 48 hours. The inhibition was statistically significant at 3-30 μg/ml of SHPS-1-Fc (**$p < 0.01$; n = 6) and at 2-20 μg/ml of rat anti-mouse CD47 mAb (**$p < 0.01$; n = 6). (C) Pertussis toxin (PT; 100 ng/ml) significantly restored the inhibition of LCs emigration by SHPS-1-Fc (3 μg/ml) treatment (**$p < 0.01$; n = 6). Error bars indicate SDs.

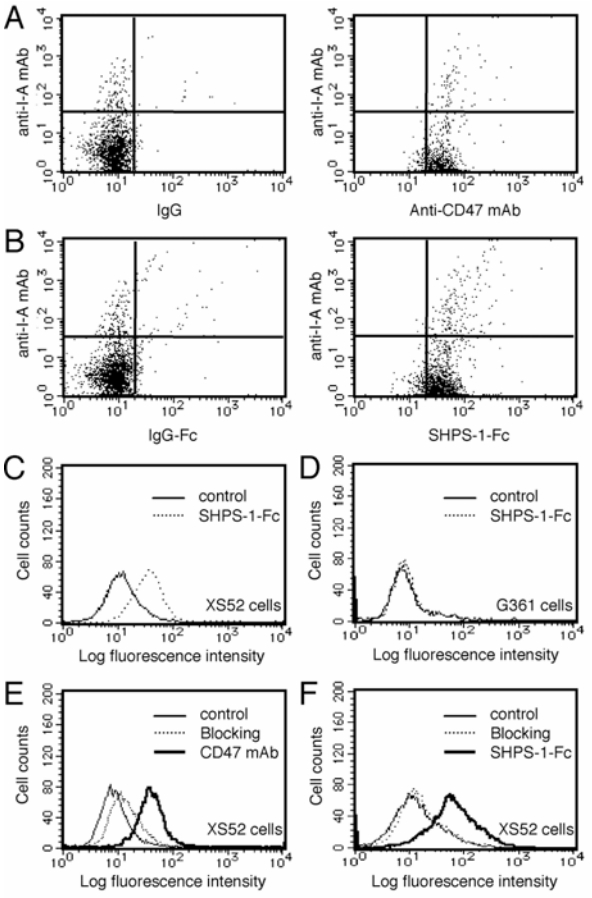
Figure 6. The chemotaxis of XS52 cells is inhibited by SHPS-1-Fc or a rat anti-mouse CD47 mAb. XS52 cells were cultured in complete RPMI supplemented with 10 ng/ml GM-CSF and 10 ng/ml IL-4 for 9 days, and chemotaxis for MIP-3β was assessed. A dose-dependent reduction in the number of migrating matured-XS52 cells was observed when (A) SHPS-1-Fc or (B) rat anti-mouse CD47 mAb was added to the culture medium. The reduction was statistically significant at 1-10 μg/ml of SHPS-1-Fc (**$p < 0.01$; n = 6) and at 0.05-5 μg/ml of anti-CD47 mAb (**$p < 0.05$; n = 6). Error bars indicate SDs. (C) Pertussis toxin (PT; 100 ng/ml) restored

the inhibition of the chemotaxis by SHPS-1-Fc treatment (**$p < 0.01$; n = 6). Error bars indicate SDs.

Figure 7. SHPS-1-Fc reduced the expression of CD80 and CD86 on epidermal LCs during skin explant culture. Epidermal explants were cultured for 24 hours with SHPS-1-Fc or control human IgG-Fc, and the expression of I-A^b (green) and CD86 (red) was then evaluated using immunofluorescence. (A) The lowest panels show the expression of I-A^b and CD86 *in situ*. CD86 is strongly expressed on the I-A^b bearing cells following explant culture incubated with IgG-Fc (right-upper, left-upper), whereas SHPS-1-Fc treatment reduced the expression of CD86 (right-middle. left-middle). Merged images are presented in the left column. (B) The area of CD86 positivity for each I-A^b-bearing cell was assessed using computer image analysis (NIH image) and data are presented as relative mean fluorescence intensity (MFI). (C) MFI for CD80 is presented in. (D) Pertussis toxin (PT; 100ng/ml) significantly reversed the effect of SHPS-1-Fc on the reduction of CD86 expression during explant culture. Data are presented as mean MFI for at least 30 cells. Error bars indicate SDs. Bar = 20 μm (**$p < 0.01$).

Figure 8. SHPS-1-Fc reduced the expression of CD80 and CD86 on emigrated LCs during skin explant culture. Epidermal explants were cultured for 16 hours with SHPS-1-Fc or control human IgG-Fc. (A, B) FACS analysis of emigrated cell suspensions shows that emigrated I-A^b-positive cells express CD80 and CD86. (A) SHPS-1-Fc treatment reduced the proportion of I-A^+ $CD80^{high+}$ cells (lower panel) compared to control human IgG-Fc (upper panel). (B) SHPS-1-Fc treatment reduced the proportion of I-A^+ $CD86^{high+}$ cells (lower panel) compared to control human IgG-Fc (upper panel).

Figure 9. Pertussis toxin reversed the inhibitory effect of SHPS-1-Fc on the CHS response. The CHS response to DNFB was significantly diminished by treatment with SHPS-1-Fc before sensitization compared with the treatment of human IgG-Fc + DNFB, whereas pretreatment with pertussis toxin (PT; 100ng, intradermally) recovered the responsiveness of CHS even with SHPS-1-Fc treatment. CHS responses are expressed as the average ear swelling, with error bars representing SDs for each group of five mice (**$p < 0.01$; n = 5).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B
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Blocking
CD47 mAb
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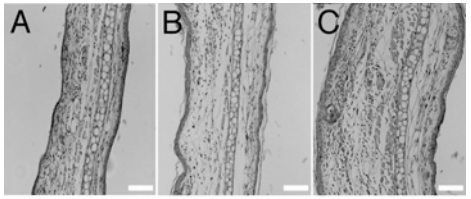
Figure 1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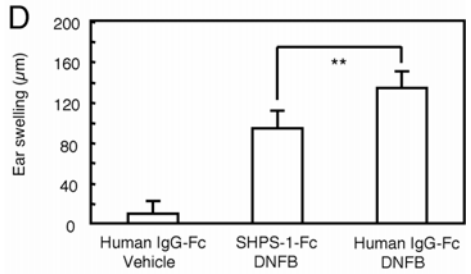
D
Ear swelling (μm)
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160
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Human IgG-Fc
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DNFB
Human IgG-Fc
DNFB

Figure 2

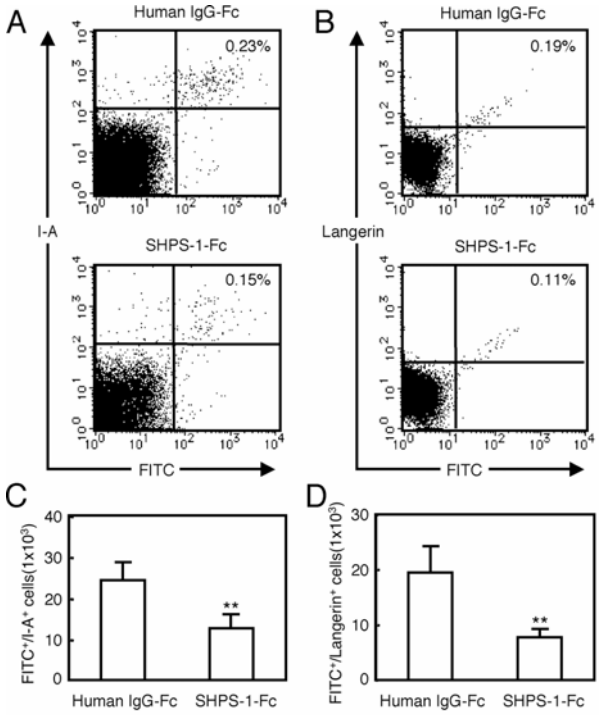
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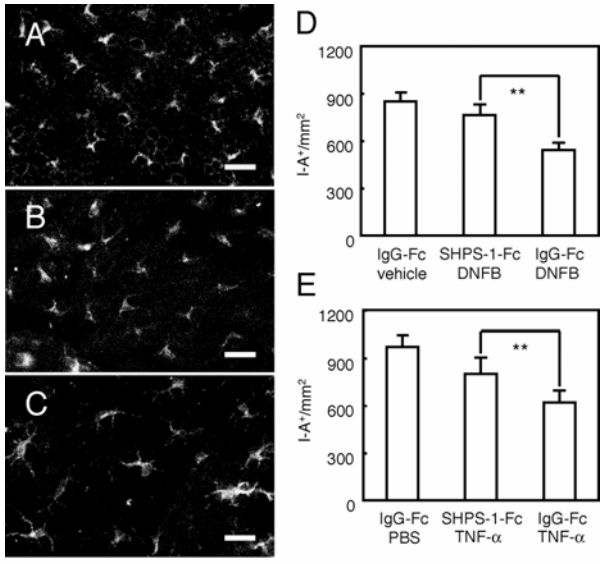
Figure 3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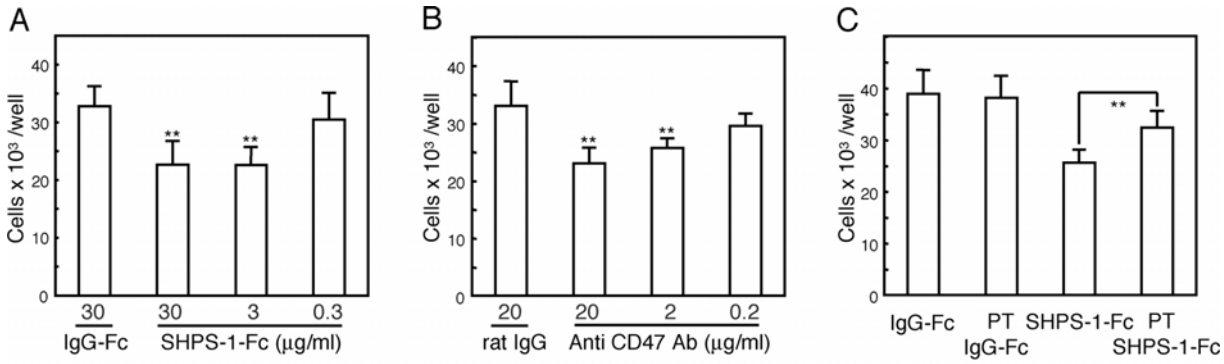
IgG-Fc TNF-α

Figure 4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IgG-Fc
SHPS-1-Fc (μg/ml)
B
Cells x 10³ /well
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rat IgG
Anti CD47 Ab (μg/ml)
C
Cells x 10³ /well
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SHPS-1-Fc
PT
SHPS-1-Fc

Figure 5

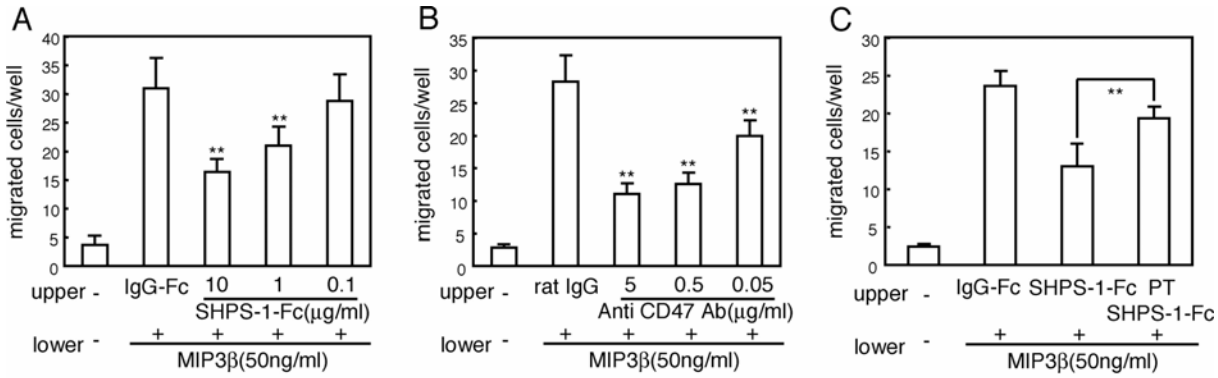
A
migrated cells/well
upper -
IgG-Fc
10
1
0.1
SHPS-1-Fc(μg/ml)
lower -
MIP3β(50ng/ml)
B
migrated cells/well
upper -
rat IgG
5
0.5
0.05
Anti CD47 Ab(μg/ml)
lower -
MIP3β(50ng/ml)
C
migrated cells/well
upper -
IgG-Fc
SHPS-1-Fc
PT
SHPS-1-Fc
lower -
MIP3β(50ng/ml)

Figure 6

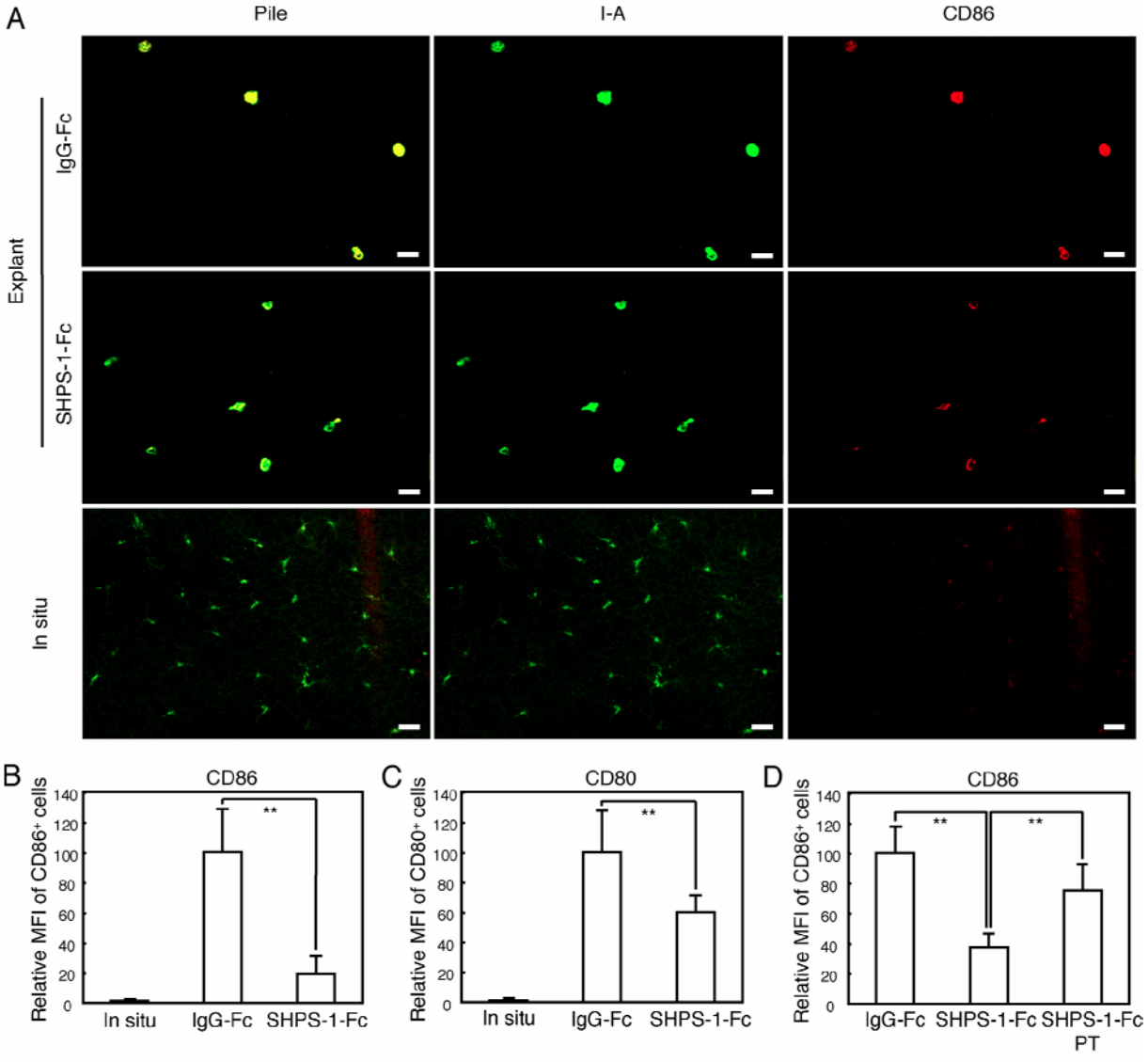
A
Pile
I-A
CD86
Explant
IgG-Fc
SHPS-1-Fc
In situ
B
CD86
Relative MFI of CD86+ cells
140
120
100
80
60
40
20
0
**
In situ
IgG-Fc
SHPS-1-Fc
C
CD80
Relative MFI of CD80+ cells
140
120
100
80
60
40
20
0
**
In situ
IgG-Fc
SHPS-1-Fc
D
CD86
Relative MFI of CD86+ cells
140
120
100
80
60
40
20
0
**
**
IgG-Fc
SHPS-1-Fc
SHPS-1-Fc
PT

Figure 7

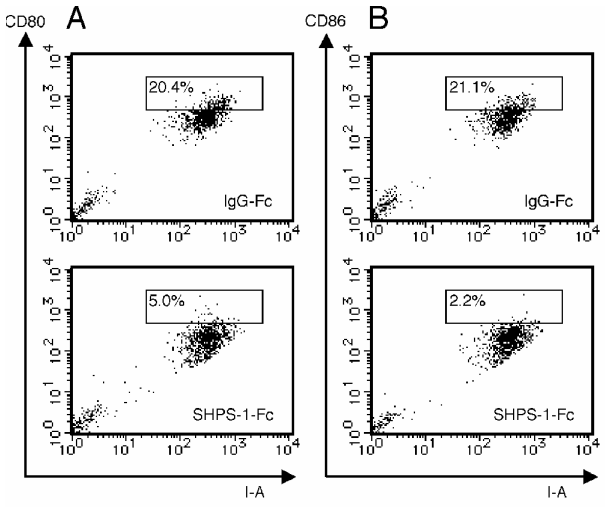

Figure 8

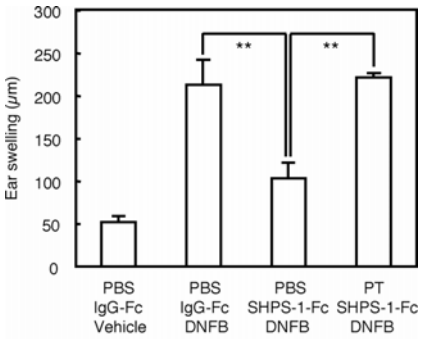
300
250
200
150
100
50
0
Ear swelling (μm)
**
**
PBS
IgG-Fc
Vehicle
PBS
IgG-Fc
DNFB
PBS
SHPS-1-Fc
DNFB
PT
SHPS-1-Fc
DNFB

Figure 9