■ 若い世代におけるHCVキャリア率は低い値を示す.

■ 「肝炎対策基本法」を基盤としてすでに感染しているキャリアへの対策が積極的に進められている.

程モデルにより推定[12]すると, 男性では50歳, 女性では60歳を過ぎるころから肝発癌率が上昇しはじめ, 60歳時点の男性の約10%, 女性の7~8%が肝癌へ, また, 70歳時点の男性の約38%, 女性の約20%が肝癌に進展すると考えられたことからも, 検査後に陽性と判定された場合には医療機関受診と継続受診が必須であるといえる.

肝炎・肝癌対策について

社会生活全般における肝炎ウイルス感染の発生要因が徐々に減少し, 若い世代におけるHCVキャリア率は低い値を示すに至っているが, 「肝炎対策基本法」(2009年12月)を基盤として, すでに感染しているキャリアへの対策, 具体的には, 肝炎ウイルス検査の推進, 肝疾患診療ネットワークの構築, 新規治療法の開発に加え, 肝炎患者の経済的負担の軽減や抗ウイルス療法の受療推進のための医療費助成制度等の事業などが積極的に進められている.

わが国では, 世界に先がけて肝炎ウイルス持続感染者の規模の把握や治療を含めた肝炎・肝癌対策が実施されてきたが, これまで行ってきた肝炎ウイルス感染の動向調査・感染防止対策を継続しつつ, 肝炎ウイルスキャリアが適切な時期に適切な治療が受けることができるよう, さらに対策を推進することが求められている.

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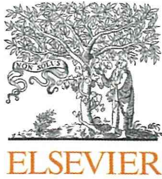

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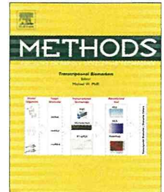

Autoimmune animal models in the analysis of the CD47–SIRPα signaling pathway

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ABSTRACT

Signal regulatory protein α (SIRPα), also known as SHPS-1/SIRPA, is an immunoglobulin superfamily protein that binds to the protein tyrosine phosphatases Shp1 and Shp2 through its cytoplasmic region and is predominantly expressed in dendritic cells and macrophages. CD47, a widely expressed transmembrane protein, is a ligand for SIRPα, with the two proteins constituting a cell–cell communication system. It was previously demonstrated that the CD47–SIRPα signaling pathway is important for prevention of clearance by splenic macrophages of red blood cells or platelets from the bloodstream. In addition, this signaling pathway is also implicated in homeostatic regulation of dendritic cells and development of autoimmunity. Here we describe the detailed protocols for methods that were used in our recent studies to study the role of the CD47–SIRPα signaling pathway in autoimmunity. We also demonstrate that hematopoietic SIRPα as well as nonhematopoietic CD47 are important for development of experimental autoimmune encephalomyelitis. Thus, we here strengthen the importance of experimental animal models as well as other methods for the study of molecular pathogenesis of autoimmunity.
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1. Introduction

Signal regulatory protein α (SIRPα) [1,2], also known as Src homology 2 domain-containing protein tyrosine phosphatase substrate-1 (SHPS-1) or BIT, is a transmembrane protein whose extracellular region comprises three immunoglobulin (Ig)-like domains and whose cytoplasmic region contains four tyrosine phosphorylation sites that mediate binding of the protein tyrosine phosphatases Shp1 and Shp2 (Fig. 1A). Tyrosine phosphorylation of SIRPα is regulated by various growth factors and cytokines as well as by integrin-mediated cell adhesion to extracellular matrix protein. Shp1 is predominantly expressed in hematopoietic cells (but also in epithelial cells) and negatively regulates various functions of these cells [3]. By contrast, Shp2 is expressed in most cell types and regulates the small GTP-binding proteins Ras and Rho, thereby contributing to the positive control of cell growth and cell migration, respectively [4]. SIRPα thus functions as a docking protein to recruit and activate Shp1 or Shp2 at the cell membrane in response to extracellular stimuli, and these protein phosphatases are thought to be important for signaling downstream of SIRPα. The extracellular region of SIRPα interacts with its ligand, CD47, through an Ig-V-like domain at the NH_2-terminus of the extracellular region of SIRPα (Fig. 1A). CD47, which was originally identified in association with $\alpha_v\beta_3$ integrin, is also a member of the Ig superfamily of proteins, possessing an Ig-V-like extracellular domain, five putative membrane-spanning segments, and a short cytoplasmic tail [5]. The binding of CD47 to SIRPα promotes the tyrosine phosphorylation of the latter protein [6,7].

Among hematopoietic cells, SIRPα is especially abundant in dendritic cells (DCs), Langerhans cells (LCs), and macrophages,

Abbreviations: BM, bone marrow; CII, type-II collagen; CD47 KO, CD47-deficient; cDCs, conventional DCs; CFA, complete Freund's adjuvant; CHS, contact hypersensitivity; CIA, collagen-induced arthritis; CNS, central nervous system; DCs, dendritic cells; DNFB, 2,4-dinitro-1-fluorobenzene; EAE, experimental autoimmune encephalomyelitis; IBD, inflammatory bowel disease; Ig, immunoglobulin; IL-10 KO, IL-10-deficient; LCs, Langerhans cells; LP, lamina propria; MOG, myelin oligodendrocyte glycoprotein; MT, mutant; PB, peripheral blood; PBS, phosphate buffered saline; RA, rheumatoid arthritis; RBCs, red blood cells; SIRPα, signal regulatory protein α; WT, wild-type.

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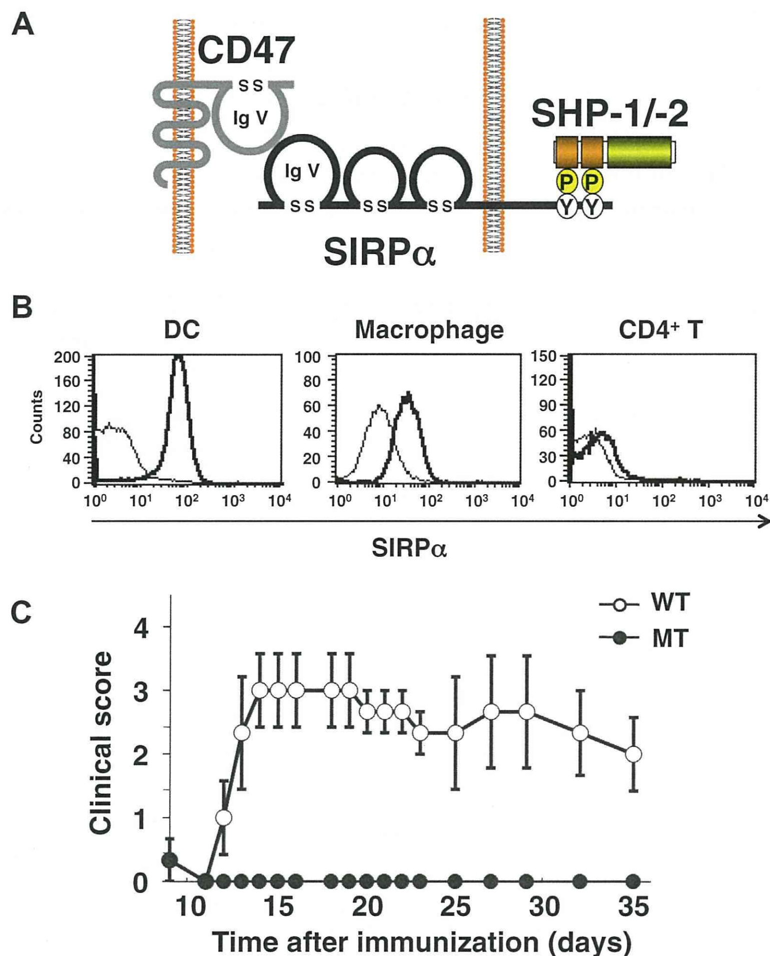

Fig. 1. The CD47–SIRPα signaling complex. (A) SIRPα is a transmembrane protein that contains three Ig-like domains in its extracellular region and a number of tyrosine phosphorylation sites in its cytoplasmic region. The tyrosine-phosphorylated sites of SIRPα bind to the protein tyrosine phosphatases, Shp1 and Shp2, and thereby activate these phosphatases. SIRPα ligand, CD47 is also a member of the Ig superfamily, possessing a V-type Ig-like extracellular domain, five membrane-spanning segments, and a short cytoplasmic tail. The N-terminal Ig V-like domain of SIRPα binds to the Ig-like domain of CD47. The binding of CD47 to SIRPα promotes the tyrosine phosphorylation of the latter protein. (B) Surface expression of SIRPα on splenic CD11c$^+$ DCs, F4/80$^+$ macrophages and CD4$^+$ T cells was analyzed by flow cytometry. (C) Minimal susceptibility of SIRPα MT mice to EAE. Time course of the clinical score of WT and SIRPα MT mice immunized with MOG(35–55) was shown. Data are means ± SE ($n = 3$) from one representative experiment.

being barely detectable in T or B lymphocytes [1,7–10] (Fig. 1B). In contrast, CD47 is expressed in a variety of hematopoietic cells including red blood cells and T cells [1,2,5]. In splenic macrophages, SIRPα, by interaction with CD47 on red blood cells (RBCs), prevents phagocytosis by the macrophages of RBCs through an SIRPα-dependent activation of Shp1 [7,11]. The SIRPα-Shp1 thereby determines both the life span of individual RBC and the number of these cells in the circulation [6,11]. The CD47–SIRPα interaction is also implicated in prevention of the clearance by splenic macrophages of transfused platelets or lymphocytes from the bloodstream [12,13].

DCs are professional antigen-presenting cells that play two distinct roles in the immune system: initiation and modulation of the immune responses of T cells to pathogens, and induction and maintenance of T cell tolerance to self-components in the periphery [14,15]. For the former role, the mature DCs present MHC–antigenic peptide complexes together with costimulatory molecules to naive CD4$^+$ Th cells, which promotes their differentiation into effector Th cells, such as IFN-γ–producing Th1 cells, IL-4-producing Th2 cells, and IL-17-producing Th17 cells [15]. In particular, Th17 cells are thought to be the principal pro-inflammatory CD4$^+$ effector T cells for development of murine models of autoimmune diseases, including experimental autoimmune encephalomyelitis, collagen-induced arthritis, and contact hypersensitivity [16,17]. SIRPα is predominantly expressed in DCs, particularly CD8α$^-$ CD11c^{high} conventional DCs (cDCs) [8,9,18]. To investigate the functional role of SIRPα in the immune regulation, we examined the susceptibility of the SIRPα mutant (MT) mice to animal models for autoimmune diseases [19–21].

2. CD47–SIRPα signaling pathway is essential for development of experimental autoimmune encephalomyelitis

2.1. Experimental autoimmune encephalomyelitis (EAE)

Multiple sclerosis is a chronic inflammatory disease of the central nervous system (CNS), characterized by discrete areas of inflammation and demyelination that can occur in multiple anatomical locations in the CNS and that can wax and wane in severity over time [22]. EAE is an animal model of multiple sclerosis, which is induced by immunization of susceptible rodent strains with myelin antigens [22]. This animal model is most commonly used for testing whether a certain molecule or a signaling pathway could be involved in induction of not only multiple sclerosis but

also of other Th1- or Th17-mediated autoimmune diseases. It should be noted by investigators that induction of EAE depends on immunogens as well as gender and genetic backgrounds of animals [23]. For instance, C57BL/6 mice are suitable to the method by the use of MOG as an immunogen that we describe below. As most gene knockout mice are generated on the 129 genetic background and then backcrossed to the genetically well-defined C57BL/6 strain, this method is quite useful for the study by the use of these gene knockout mice. In contrast, from the injection of antigens, it takes approximately one month to complete the observation of the responses of immunized mice. The cost for the experiments (in particular, for synthetic peptides such as MOG) is expensive compared to other animal models described in the following sections.

2.1.1. Protocol for induction of EAE [19]

Male C57BL/6 mice are subcutaneously injected (into the base of the tail and rump) with a total of 100 μg of a peptide derived from myelin oligodendrocyte glycoprotein [MOG(35–55)] (MEVGWYRSPFSRVVHLYRNGK) in complete Freund's adjuvant (CFA) containing 400 μg of heat-inactivated *Mycobacterium tuberculosis* (Difco). Pertussis toxin (200 ng) (List Biological Laboratories) is also injected intravenously on day 0 and 2. Animals are observed daily for clinical signs of EAE for up to 35 days after immunization, and neurological effects were quantified on an arbitrary clinical scale: 0, no disease; 1, limp tail; 2, hindlimb weakness; 3, total hindlimb paralysis; 4, hind- and forelimb paralysis; and 5, death. The mean clinical score was calculated by averaging the scores of all mice in each group, including animals that did not develop EAE.

We generated mice that express a MT version of SIRPα that lacks most of the cytoplasmic region [7,11]. This MT protein does not undergo tyrosine phosphorylation or form a complex with Shp1 or Shp2. Furthermore, the cellular abundance of the MT protein is markedly reduced compared with that of the full-length protein in wild-type (WT). Given the importance of the cytoplasmic region of SIRPα for signaling by this protein, the function of SIRPα is thought to be eliminated in the MT mice. SIRPα MT mice were backcrossed to the C57BL/6 background for five generations. We previously showed that SIRPα MT mice were markedly resistant to the development of EAE [19]. Indeed, we here confirmed such resistance of SIRPα MTmice to EAE in the new experiment (Fig. 1C). The MOG(35–55)-induced proliferation of, and production of IL-17 by, T cells from immunized SIRPα MT mice were also reduced compared with those apparent for WT cells [19]. Thus, it was suggested that SIRPα on DCs is important for induction by DCs of the antigen-specific Th cells producing IL-17 and development of EAE.

2.2. Bone marrow (BM) chimeras

We next examined if SIRPα in DCs or macrophages is indeed important for induction of EAE. To this end, we generated BM chimeras. The experiments by the use of BM chimera are often used in the immunology research, and here we tried to see whether hematopoietic or nonhematopoietic SIRPα is essential for development of EAE.

2.2.1. Protocol for generation of BM chimeras [8]

Recipient B6-Ly5.1 mice were subjected to lethal irradiation (9.5 Gy) and then injected intravenously with 5×10^6 BM cells obtained from Ly5.2$^+$ WT or SIRPα MT mice (WT or MT BM → Ly5.1). Conversely, recipient Ly5.2$^+$ WT and SIRPα MT were subjected to lethal irradiation and injected intravenously with 5×10^6 BM cells obtained from B6-Ly5.1 mice (Ly5.1 BM → WT or MT). More than 6 weeks after transplantation, 50–100 μl of peripheral blood (PB) was taken from the recipient animals, stained for FITC-conjugated anti-Ly5.2 antibody (BD Biosciences), and then percentage of donor-derived cells (Ly5.2-positive) in PB was measured by flow cytometry (FACS Calibur, BD Biosciences). The recipients in which >90% of PB cells was reconstituted with donor-derived cells were used for induction of EAE.

SIRPα MT BM → Ly5.1 chimeras were markedly resistant to EAE, compared with WT BM → Ly5.1 chimeras (Fig. 2A). In contrast, the susceptibility to EAE did not differ between Ly5.1 BM → SIRPα MT and Ly5.1 BM → WT chimeras (Fig. 2B). These results thus suggest that hematopoietic SIRPα, but not nonhematopoietic SIRPα, is important for development of EAE.

2.3. CD47-deficient mice

We also examined the importance of CD47 for induction of EAE. CD47-deficient (CD47 KO) mice were described previously [24] and were backcrossed to the C57BL/6 background for >10 generations. Similar with SIRPα MT mice, we found that CD47 KO mice were quite resistant to EAE (Fig. 3A). CD47 is expressed widely in both hematopoietic cells and nonhematopoietic cells [1,5]. We therefore next examined whether nonhematopoietic CD47 is important for induction of EAE. When irradiated CD47 KO mice were reconstituted with BM cells from Ly5.1 WT mice, such chimera mice were also resistant to EAE, compared with Ly5.1 WT BM → WT chimeras (Fig. 3B). These data thus indicated that nonhematopoietic CD47 is, at least in part, important for development of EAE. The transfer of BM cells from CD47 KO mice into WT reci-

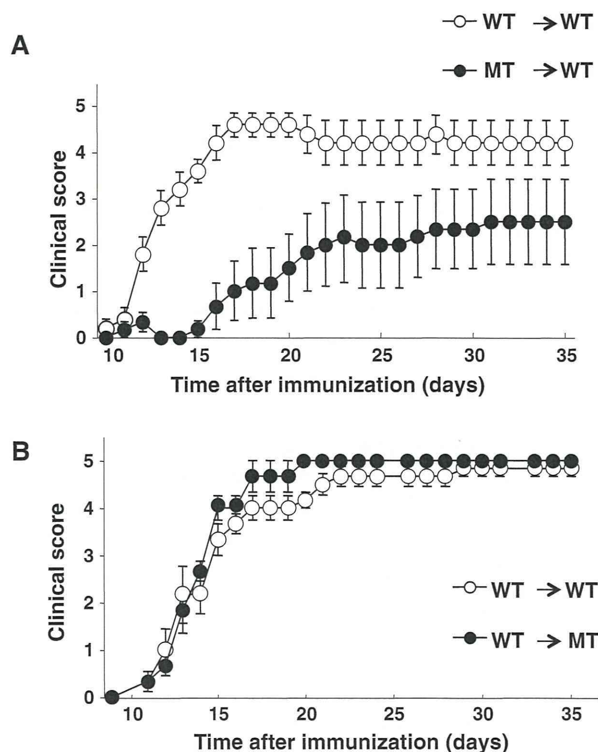

Fig. 2. Importance of hematopoietic SIRPα for development of EAE. B6-Ly5.1 mice were lethally irradiated and then reconstituted with 5×10^6 BM cells from WT or SIRPα MT mice (A). Conversely, WT or SIRPα MT mice were lethally irradiated and reconstituted with 5×10^6 BM cells from B6-Ly5.1 mice (B). Six to eight weeks after transplantation, chimeras were subjected to EAE. Time courses of the clinical score of immunized mice are shown. Data are means ± SE (A; WT → WT ($n = 5$), MT → WT ($n = 6$). B; WT → WT ($n = 6$), WT → MT ($n = 6$)) and representative of (A) three and (B) two independent experiments.

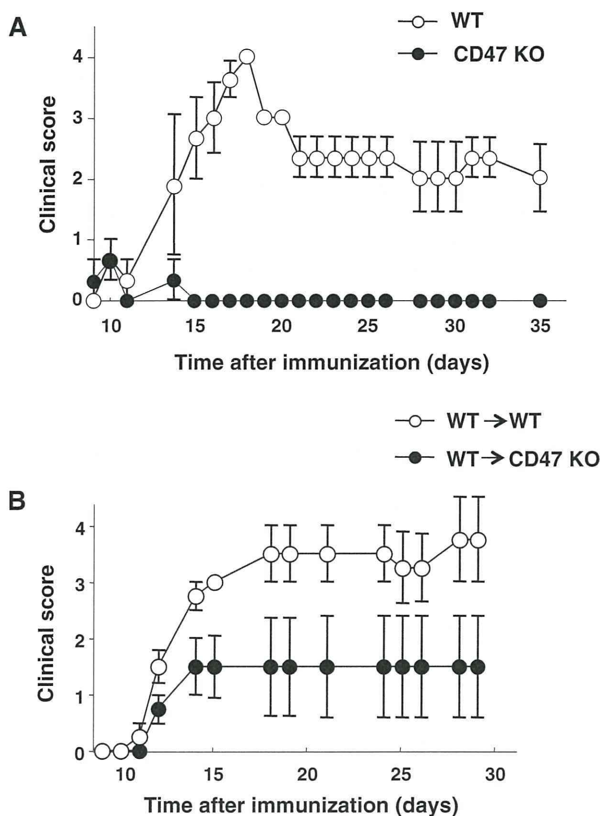

Fig. 3. Importance of CD47 for development of EAE. Time course of the clinical score of immunized CD47 KO (A) or BM chimeras (Ly5.1 WT BMs → WT or CD47 KO) (B) was shown. Data are means ± SE (A; $n = 3$, B; $n = 4$).

pient mice results in the rapid elimination of these cells, likely as a result of the lack of prevention by CD47–SIRPα interaction of phagocytosis by splenic macrophages [13]. Indeed, we failed to reconstitute the BM of irradiated Ly5.1 WT mice with BM cells from CD47 KO mice (data not shown).

3. Importance of CD47–SIRPα signaling pathway for other experimental autoimmune diseases

3.1. Collagen-induced arthritis (CIA)

Rheumatoid arthritis (RA) is autoimmune in nature and characterized by chronic inflammation of the synovial tissues in multiple joints [25,26]. Although the molecular basis for its etiopathogenesis has not been fully elucidated, the sequence of events in the pathogenesis of the disease, as well as the end-stage effector mechanisms, are clarified [25,26]. In the initial inflammatory lesions, $CD4^+$ Th cells are thought to play an essential role of initiation of subsequent inflammatory responses. It is also known that the Th17 cell is the main pro-inflammatory $CD4^+$ effector T cell involved in CIA [27]. The most common animal model for RA is type-II collagen (CII)-induced arthritis [28]. CIA model is restricted to mice bearing the MHC class II $H\text{-}2^q$ haplotype, such as DBA/1 background. However, the method we used here is that CIA is induced by immunization of C57BL/6 ($H\text{-}2^b$) mice with chick CII together with excess amount of heat-inactivated *M. tuberculosis* [20,28]. From the injection of antigens, it takes a longer time (approximately 2–3 months) to complete the observation of the responses of immunized mice. The cost for the experiments is less expensive compared to the EAE as described above.

3.1.1. Protocol for induction of CIA [20]

C57BL/6-background mice (6- to 12-week-old) were immunized intradermally with a total of 100 μl emulsion containing 100 μg chick CII (Sigma–Aldrich) emulsified in CFA containing 250 μg of heat-inactivated *M. tuberculosis* at several sites into the base of the tail and the same injection was repeated at day 21. For DBA/1-background mice, animals were similarly immunized except for the use of a total of 150 μl emulsion containing 200 μg bovine CII (Collagen Research Center, Tokyo, Japan) emulsified in CFA containing 100 μg of heat-inactivated *M. tuberculosis*. The animals were assessed for redness and swelling of limbs and clinical score allocated for each mouse twice per week. The clinical severity of arthritis was quantified according to the following scoring system: 0, no change; 1, swelling in digital joint; 2, swelling of paws; 3, severe swelling of digital joint and the entire paw. Each paw was graded, so that each mouse could achieve a maximum score of 12.

We found that SIRPα MT mice were markedly resistant to CIA [20]. Indeed, ~60% of C57BL/6-background WT mice developed typical CIA ~45 days after immunization. In contrast, none of SIRPα MT mice developed CIA [20]. Because C57BL/6 mice are relatively resistant to CIA compared to DBA/1 mice [20], we thus next crossed SIRPα MT mice with DBA/1 mice and examined the development of CIA in such DBA/1-background animals. All of DBA/1 WT mice immunized developed CIA ~25 days after immunization. In contrast, only ~60% of DBA/1-backcrossed SIRPα MT mice developed CIA. Consistently, the serum levels of both IgG and IgG2a antibodies specific to CII in immunized SIRPα MT mice were markedly reduced compared with those apparent for WT mice [20]. In addition, the CII-induced proliferation of, and production of IFN-γ, IL-6 and IL-17 by, T cells from immunized SIRPα MT mice were reduced compared with those apparent for WT cells [20]. Thus, these results suggest that SIRPα is important for development of CIA as well as induction of Th17 cells.

3.2. Contact hypersensitivity (CHS)

Given that LCs and dermal DCs express SIRPα [10,29], we also evaluated the importance of SIRPα and CD47, as well as that of the interaction between these two proteins, in the development of contact hypersensitivity (CHS). CHS is an inflammatory immune response of skin that is induced by the local application of haptens [30]. LCs and dermal DCs are professional antigen presenting cells and play a central role in the induction of CHS [30]. IL-17 production by $CD4^+$ Th cells known as Th17 cells was shown to be essential for development of CHS like other autoimmune diseases [31,32]. Indeed, IL-17–deficient mice manifest reduced susceptibility to CHS [32]. C57BL/6 mice are susceptible to CHS that is induced by the protocol described below. From the injection of antigens, the examination takes only 1 week and the cost for the experiments is inexpensive compared to other model described above, indicating that this model could be simple and worth testing first compared with other models described above.

3.2.1. Protocol for induction of CHS [21]

Female mice (8- to 12-week-old) were sensitized by the application of 50 μl of 2,4-dinitro-1-fluorobenzene (DNFB) (Sigma–Aldrich) (0.5% in acetone/olive oil, 4:1, v/v) on their shaved abdomen on day 0. On day 5, 10 μl of 0.2% DNFB were applied to the dorsal and ventral surfaces of the right ear; vehicle (acetone/olive oil) was applied to the left ear. Ear thickness was measured in a blinded manner with a digimatic micrometer (Mitutoyo, Kawasaki, Japan) 24 h after antigen challenge; an increase in the ear thickness (swelling) was defined as the difference in thickness between before and 24 h after DNFB-treatment.

The CHS response to DNFB was significantly reduced in SIRPα MT mice compared with that in WT mice [21], suggesting that SIRPα is essential for development of CHS. In contrast, development of CHS was not impaired in CD47 KO mice [21].

3.3. Importance of SIRPα in the intestinal immunity and IL-10–deficient mice

The intestinal immune system of mammals is responsible for maintaining immunologic tolerance to food antigens and commensal organisms as well as for the recognition of harmful pathogens and their elimination by humoral or cellular immune responses [33]. Deregulation of these dual functions results in uncontrolled inflammatory disorders such as inflammatory bowel disease (IBD) in humans [34]. IL-10–deficient (IL-10 KO) mice are animal model of IBD; they manifest spontaneous colitis that resembles the colitis in human IBD such as Crohn's disease or ulcerative colitis [35,36]. IL-10 is thought to inhibit various types of cells that promotes immune responses [37]. Both Th1 cells and Th17 cells are implicated in the development of spontaneous colitis associated with IL-10 deficiency [36,38,39].

DCs or macrophages are thought to be key regulators of the intestinal immune system [40]. These cells are widely distributed in intestinal lymphoid organs including Peyer's patches and mesenteric lymph nodes, but they also reside in the connective tissue underlying the intestinal epithelium, the lamina propria (LP). However, the function of SIRPα in intestinal immunity had not been well characterized. We thus developed methods for efficient isolation of LP cells to study the expression of SIRPα in DCs or macrophages, as well as the functional role of this protein, in the LP.

3.3.1. Protocol for isolation of LP cells [40]

The small intestine and colon were excised from adult mice and freed of mesentery; for the small intestine, Peyer's patches were also removed. The small intestine or colon was then opened longitudinally and washed with phosphate buffered saline (PBS) to remove fecal content. The tissue was cut into pieces 1.5–2 cm in length, transferred to a 50-ml conical tube or 500-ml plastic bottle, and shaken for three or four 15-min periods at room temperature in PBS containing 5 mM EDTA. The muscle layer was removed from the remaining tissue, which was then washed with PBS, minced, and incubated for 20 min at 37 °C in Hank's balanced salt solution containing collagenase type IV (Sigma–Aldrich) at 1 mg/ml. The resulting cell suspension was passed through a 70-μm cell strainer (BD Biosciences), and the cells were collected by centrifugation at 340*g*. The cells were then suspended in PBS containing 40% (v/v) Percoll (GE Healthcare, Piscataway, NL, USA), gently overlaid on 75% Percoll in PBS, and centrifuged at 700*g* for 20 min at room temperature. Cells at the interface of the two layers were collected, washed, and studied as LP cells.

We showed that SIRPα is expressed in $CD11c^{hi}$ $CD11b^{hi}$ DCs (R2 subset) as well as in $CD11c^{int}$ $CD11b^{int}$ macrophages (R3) and $CD11c^{int}$ $CD11b^{hi}$ eosinophils (R4) in LP cells [40]. However, the expression level of SIRPα was low or minimal in $CD11c^{hi}$ $CD11b^{lo}$ DCs (R1). R2-LPDCs are thought to promote the differentiation of antigen-specific Th17 cells and Th1 cells as well as IgA class switch recombination in B cells [41,42]. Indeed, the *in vitro* flagellin-stimulated production of IL-17 or IFN-γ by LP cells of SIRPα MT mice was markedly decreased compared with that observed with WT cells [40]. Thus, SIRPα on $CD11c^{+}$ cells such as R2-LPDCs is likely important for the flagellin-stimulated production of IL-17 and IFN-γ in cultures of LP cells.

To investigate the function of SIRPα in development of colitis, we crossed SIRPα MT mice with IL-10 KO mice on the C57BL/6 background (B6.129P2-$Il10^{tm1Cgn}$/J) (obtained from The Jackson Laboratory) and then examined the susceptibility of SIRPα MT mice to spontaneous colitis associated with IL-10 deficiency [40]. We first monitored disease activity for colitis, which was scored on the basis of stool consistency, blood in the stool, and anorectal prolapse, in IL-10 KO mice and SIRPα MT/IL-10 KO mice. Consequently, we found that the disease activity in SIRPα MT/IL-10 KO mice at either 10 or 16 weeks of age was markedly reduced compared with that apparent in IL-10 KO mice. The histological analysis also demonstrated that SIRPα MT mice are resistant to the development of colitis induced by IL-10 deficiency [40]. The frequency of $CD4^{+}$ T cells producing IFN-γ as well as IL-17 was significantly decreased in the colonic LP of SIRPα MT/IL-10 KO mice compared with that in IL-10 KO mice [40].

4. Discussion

We have described herein the detailed protocols for animal models of autoimmunity, such as EAE, CIA, and CHS. These animal models are most frequently used for testing whether a certain molecule or a signaling pathway likely participates in induction of Th1- or Th17-mediated autoimmune diseases. We have also detailed the genetic backgrounds and how each model compares in terms of cost and time in relation to experimental outcome. From our experiments using these animal models for Th17-mediated autoimmunity, we have shown that SIRPα MT mice were markedly resistant to the development of autoimmunity [19–21,40]. Based on these observations, it is highly likely that the CD47–SIRPα signaling pathway is required for development of the Th17-mediated autoimmunity. In addition to the results based on the use of BM chimeras, we also demonstrated that hematopoietic SIRPα, but not non-hematopoietic SIRPα, is important for development of EAE. Given that SIRPα is predominant in DCs or macrophages among various blood cells, SIRPα expressed on these hematopoietic cells is likely indispensable for EAE. In particular, we previously demonstrated that the number of $CD8\alpha^{-}$ $CD4^{+}$ cDCs is selectively reduced in secondary lymphoid tissues of SIRPα MT mice [8]. Moreover, such reduction of cDCs is also observed in SIRPα MT BM → WT chimeras [8]. Thus, the resistance of SIRPα MT mice to EAE might be attributable to impairment of DC homeostasis.

Similar to SIRPα MT mice, we have demonstrated that CD47 KO mice are also resistant to EAE. Moreover, the BM chimera experiments suggest that CD47 in nonhematopoietic cells is, at least in part, required for EAE. Such observations are consistent with the recent report by Han et al. [43]. Given that CD47 is a ligand for SIRPα, trans interaction of CD47 (on nonhematopoietic cells) with SIRPα (on hematopoietic cells such as DCs) is indeed essential for development of EAE. However, the precise mechanism by CD47–SIRPα signaling pathway that regulates development of EAE as well as DC homeostasis remains poorly understood. Thus, future study should be directed to clarify such issues by the use of mice that are deleted with either SIRPα or CD47 in a cell-type specific manner.

In conclusion, the use of experimental animal models that we described here, combined with other conventional methods for immunology such as BM chimeras, are indispensable tools in the study of the molecular pathogenesis of autoimmunity.

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The BALB/c-specific polymorphic SIRPA enhances its affinity for human CD47, inhibiting phagocytosis against human cells to promote xenogeneic engraftment

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It has been shown that in xenotransplantation of human cells into immunodeficient mice, the mouse strain background is critical. For example, the nonobese diabetic (NOD) strain is most efficient, the BALB/c is moderate, and the C57BL/6 is inefficient for human cell engraftment. We have shown that the NOD-specific polymorphism of the signal regulatory protein-alpha (*Sirpa*) allows NOD SIRPA to bind human CD47, and the resultant "don't eat me" signaling by this binding prevents host macrophages to engulf human grafts, thereby inhibiting rejection. Here we tested whether the efficient xenotransplantation capability of the BALB/c strain is also mediated by the SIRPA-CD47 self-recognition system. BALB/c SIRPA was capable of binding to human CD47 at an intermediate level between those of C57BL/6 SIRPA and NOD SIRPA. Consistent with its binding activity, BALB/c-derived macrophages exhibited a moderate inhibitory effect on human long-term culture-initiating cells in in vitro cultures, and showed moderate phagocytic activity against human hematopoietic stem cells. The increased affinity of BALB/c SIRPA for human CD47 was mounted at least through the BALB/c-specific L29V SNP within the IgV domain. Thus, the mouse strain effect on xenogeneic engraftment might be ascribed mainly to the binding affinity of strain-specific polymorphic SIRPA with human CD47. This information should be useful for developing a novel immunodeficient strain with superior efficiency for xenogeneic transplantation of human cells. **© 2014 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Immunodeficient mice are widely used as hosts for the xenotransplantation models to evaluate biological activity of human hematopoietic stem cells (HSCs) [1–4]. In this setting, T, B, and NK cells play a critical role in human cell rejection; therefore, to achieve human cell engraftment, the lymphoid system in mouse recipients must be abrogated. In developing immunodeficient mouse lines for human cell xenotransplantation, introduction of the *scid* mutation of *Pkrdc* [5–7] or depletion of recombination activating gene 1 or 2 (*Rag1* or *Rag2*) [8,9] has been used to abrogate T and B cell development, whereas depletion of interleukin (IL) 2 receptor common gamma chain subunit (*Il2rg*) [10–12], or of beta-2-microglobulin (*B2m*) [13–15] has been used to abrogate NK cells or their functions.

In addition to abrogation of the lymphoid system, some strain-specific factors might affect the efficiency of human hematopoietic engraftment in mice [16]. For example, the BALB/c strain with *scid* mutation was originally used to

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reconstitute human immune system [6], and mouse lines of this strain with other immunodeficient abnormalities have been developed [17–19]. The nonobese diabetic (NOD) strain is currently more popular because its SCID mouse line (NOD-*scid*) could support higher levels of human hematopoietic engraftment [7] as compared with the BALB/c SCID. In contrast, the *scid* mutant strains with other than BALB/c and NOD backgrounds cannot support human cell engraftment [20]. Thus, immunodeficient NOD mouse models have been become the gold standard in xenotransplantation assays, and NOD-*scid* $Il2rg^{null}$ (NSG/NOG) [10,11] and NOD.$Rag1^{null}Il2rg^{null}$ [21] strains are currently the most popular mouse lines used for human cell xenotransplantation.

We have recently reported that a highly efficient human cell engraftment seen in the NOD line is attributable to the NOD-specific polymorphism of signal regulatory protein-alpha (*Sirpa*) [16]. We found that human hematopoiesis was maintained in vitro for a long-term on a bone marrow stromal layer from NOD, but not from other mouse strains [16], and that this culture system appeared to reflect the strain-related efficiency for human cell transplantation. The positional genetics enabled us to find that the genetic determinant for maintenance of human long-term culture-initiating cells (LTC-ICs) is located within the insulin-dependent diabetes (*Idd*)-13 locus [16], where only the *Sirpa* gene has the NOD-specific polymorphism.

SIRPA is a transmembrane protein expressed in macrophages, myeloid cells, and neurons, and it contains three immunoglobulin (Ig)-like domains within the extracellular region. Through its IgV-like domains, SIRPA interacts with its ligand CD47, which is ubiquitously expressed [22,23]. The binding of cell-surface CD47 with SIRPA on macrophages' surface provokes inhibitory signals for phagocytosis, called "don't eat me" signals [24–26]. NOD has a unique SIRPA IgV domain that can bind to human CD47 [16], preventing macrophages from engulfing human HSCs. We then developed the C57BL/6.$Rag2^{null}$-$Il2rg^{null}$ strain harboring NOD-specific *Sirpa*, named the BRGS mouse [27]. The efficiency of human cell engraftment in BRGS mice was equal to that of NOD.$Rag2^{null}$-$Il2rg^{null}$ mice [27]. These findings clearly show that in addition to depletion of lymphocytes, inactivation of phagocytosis via the CD47-SIRPA interaction is one of the critical determinants to establish an efficient xenogeneic transplantation system.

The question is whether the strain effect for human cell engraftment efficiency can be explained solely by the SIRPA. In the BALB/c strain, the BALB/c.$Rag1^{null}Il2rg^{null}$ mice are moderately efficient for human cell transplantation, in direct comparison with NSG and NOD.$Rag2^{null}Il2rg^{null}$ mice, irrespective of the transplantation protocol or age of recipient mice [18]. In addition, it has been shown that BALB/c.$Rag2^{null}Jak3^{null}$ mice but not C57BL/6.$Rag2^{null}Jak3^{null}$ mice supported human HSC engraftment [19]. These data led us to test the SIRPA polymorphic status and its functions in the BALB/c strain.

In this study, we found that the BALB/c strain possesses a unique *Sirpa* polymorphism that enables the BALB/c SIRPA IgV domain to bind human CD47 moderately. These data suggest strongly that the mouse strain effect on xenotransplantability is attributable mainly to the binding capability of strain-specific SIRPA with human CD47.

Materials and methods

Mice

C57BL/6 and C3H mice were purchased from CLEA Japan; 129, ICR, and BALB/c mice were purchased from Kyudo Japan (Saga, Japan). Nonobese diabetes-resistant (NOR) mice homozygous for NOD-derived *Idd13* (NOR.NOD-*Idd13*) were purchased from the Jackson Laboratory. The NOR strain is a recombinant inbred strain that is 88% identical to the NOD strain, differing only at four *Idd* loci (*Idd4*, *Idd5*, *Idd9*, and *Idd13*). All mice were bred and maintained in individual ventilated cages at the Kyushu University Animal facility (Fukuoka, Japan) and fed with autoclaved food and water. All experiments were conducted following the guidelines of the institutional animal committee of Kyushu University.

Human cord blood samples

Cord blood (CB) samples from full-term deliveries were obtained from healthy volunteers who had provided informed consent (Japanese Red Cross Kyushu Cord Blood Bank, Fukuoka, Japan). The Institutional Review Board of Kyushu University Hospital approved all of the associated experiments.

Preparation of mouse macrophages

Mouse peritoneal macrophages and bone marrow cells were obtained as previously described [26,27]. Mouse bone marrow–derived macrophages were obtained by the culture of bone marrow cells with recombinant mouse granulocyte-monocyte colony-stimulating factor (40 ng/ml; R&D Systems, Minneapolis, MN, USA) [26].

Preparation of soluble human CD47-Fc and mouse CD47-Fc fusion protein

CHO-Ras-human CD47-Fc and CHO-Ras-mouse CD47-Fc hybridoma cells were established previously [28]. Human CD47-Fc and mouse CD47-Fc fusion protein were purified from culture supernatants of these hybridoma cells by column chromatography on a HiTrap Protein-G HP column (GE Healthcare Bio-Sciences Japan, Tokyo, Japan).

Antibodies and cell staining

To analyze mouse SIRPA and human CD47-Fc binding, mouse macrophages and HeLa cells were stained with FITC-conjugated CD11b (Beckman Coulter, Fullerton, CA, USA), PE-conjugated rat anti-Mouse SIRPA (BD Pharmingen, San Diego, CA, USA) and biotinylated human CD47-Fc plus allophycocyanin-conjugated streptavidin. The $CD34^{+}CD38^{-}$ subfraction in human CB samples was identified by staining lineage-depleted (Lin^{-}) CB cells with FITC-conjugated anti-CD34 (581/CD34) and PE-conjugated anti-CD38 (HIT2; BD Biosciences, San Jose, CA,

USA). The cells were analyzed and sorted with a FACSAria cell sorter (BD Biosciences) [29,30].

DNA sequencing of the Sirpa IgV *domain*

DNA sequencing of the mouse *Sirpa* IgV domain was performed by PCR amplification of cDNA prepared from the peripheral blood of BALB/c, 129, ICR, and C3H mice. The oligonucleotide primers had been designed specifically for each strain (Supplementary Table E1, online only, available at www.exphem.org).

SIRPA-CD47 binding assay

Confluent mouse bone marrow–derived macrophages or HeLa cells infected with lentiviral vectors expressing mouse SIRPA were incubated in a 96-well plate in the presence of increasing concentrations of purified human CD47-Fc fusion protein for 30 min at 37°C, and then incubated with horseradish peroxidase–conjugated goat polyclonal antibody specific for the Fcγ fragment of human IgG (Jackson ImmunoResearch, West Grove, PA, USA) for 30 min at 4°C. Human CD47-Fc fusion protein binding was determined using a peroxidase activity assay, and absorbance was measured at 490 nm on a microplate reader. Nonlinear regression analysis was performed to calculate Kd using the KaleidaGraph analysis program.

SHP-1 immunoblot analysis

The bone marrow–derived macrophages were incubated with purified human CD47-Fc fusion protein for 1 hour at 37°C. Jurkat cells (a human T cell acute lymphoblastic leukemia cell line; American Type Culture Collection, Manassas, VA, USA), which were used as a positive control, were incubated with human epidermal growth factor (200 ng/mL; R&D Systems) for 10 min at 37°C. The cells were lysed and incubated with rabbit polyclonal antibodies for mouse SIRPA (2 µg/mL; Abcam, Cambridge, MA, USA) for the purification of SIRPA protein. Next, cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham, Uppsala, Sweden) as described previously [31]. Membranes were probed using rabbit polyclonal antibody for phosphorylated tyrosine 536 on mouse Src homology 2 domain-containing protein tyrosine phosphatase (SHP)-1 (Full Moon BioSystems, Sunnyvale, CA, USA) and visualized using an ECL detection system (GE Healthcare).

In vitro mouse macrophage phagocytosis of human hematopoietic stem cells

The phagocytic activity of mouse macrophages against the human $CD34^+CD38^-$ population isolated from CB samples was evaluated in vitro as described previously [26,27,32]. In brief, mouse peritoneal-derived macrophages or C57BL/6 bone marrow-derived macrophages lentivirally transduced with strain-specific or mutated *Sirpa* were incubated at a density of 1.0×10^4 cells in 200 µL of RPMI 1640 medium with mouse IFN-γ (100 ng/mL; R&D Systems) for 24 hours and in LPS (0.3 µg/µl) for 1 hour. Isolated 1.5–3.5 $\times$ 10^4 human CB cells were opsonized with CD34 antibody (sc-19621; Santa Cruz, CA, USA) and then added to the macrophages. Two hours after coincubation with the macrophages and target cells, the phagocytic index was calculated using the following formula: Phagocytic index = Number of ingested cells / (Number of macrophages / 100).

Cloning and mutagenesis of mouse Sirpa *for insertion into lentiviral vectors*

The *Sirpa* coding sequence (CDS) from cDNA of C57BL/6, NOD, and BALB/c mice was amplified by PCR and cloned downstream of an EIFα promoter in a third-generation CEP lentiviral vector backbone containing a reporter gene encoding GFP driven by the promoter of the human gene encoding phosphoglycerate kinase. The CEP lentiviral vectors were provided by Dr. John E. Dick (University of Toronto, Canada). A replacement of Leu at position 29 with Val (L29V) was introduced into lentiviral vector expressing C57BL/6 *Sirpa* using the DpnI method [33].

Sirpa *lentiviral infection*

Viruses pseudotyped with the vesicular stomatitis G protein were generated by transient infection with lentiviral vectors expressing C57BL/6, NOD, BALB/c-*Sirpa* or C57BL/6-*Sirpa* L29V as described previously [16]. Viruses at a multiplicity of infection of 10 or 1–2 were added to flasks of C57BL/6 bone marrow–derived macrophages or HeLa cells, respectively. Six days later, infected macrophages were sorted with an FACSAria (BD Biosciences) to obtain GFP^+CD11b^+ cells (Fig. 2B). Uninfected control macrophages were sorted to obtain GFP^-CD11b^+ cells. On day 8, infected HeLa cells were sorted to obtain GFP^+SIRPA^+ cells. Purified infected macrophages were used for the LTC assay; purified infected HeLa cells were used for the SIRPA-CD47 binding assay.

Long-term culture of human hematopoietic cells with mouse macrophages

For the long-term culture of human hematopoietic cells on MS-5 cells, unirradiated MS-5 cells were seeded into 96-well tissue culture plates (2×10^3 cells/well) as described previously [16]. Peritoneal macrophages or bone marrow–derived macrophages were seeded at doses of 2 $\times$ 10–2 $\times$ 10^3 cells per well. Next, 2 $\times$ 10^3 human Lin^- CB cells were added to each well. After 4–5 weeks, cells were harvested and plated for a progenitor assay as described previously [16]. The number of colony-forming cells was counted after 2 weeks of culture. LTC-ICs were defined as colony-forming cells surviving after 4–5 weeks of stromal culture.

Statistical analysis

For the comparison of SIRPA-CD47 binding capabilities among strains, the Kd values in each group were estimated using the nonlinear least squares method. The F values for comparison were obtained using the least-squares function on JMP version 9 software. The binding capability of mouse SIRPA-human CD47 at a concentration of 500 nM was estimated for each group; mean values were compared using the Dunnett's method. The phagocytic index for each strain was normalized to control values. Differences among groups in the long-term chimeric culture assay were evaluated using a normal test based on a specific nonlinear model.

Results

SIRPA on BALB/c macrophages shows modest binding capability to human CD47

We first evaluated whether SIRPA expressed on the surface of macrophages can bind to human CD47. As shown in Figure 1A, a high level of SIRPA expression was seen in $CD11b^+$ macrophages in any of the C57BL/6, NOD, and