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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

A running title; Sirpa directs human cell engraftment in BALB/c strain

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Abstract (219 words)

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- α (*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 to develop a novel immunodeficient strain with superior efficiency for xenogeneic transplantation of human cells.

Key words: Sirpa, xenogeneic engraftment, BALB/c, immunodeficient mice, phagocytosis

Introduction

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, and 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 employed 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 reconstitute human immune system [6], and mouse lines of this strain with other immunodeficient abnormalities have been developed [17-19]. The non-obese 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 to 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 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- α (*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 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.

Here, we found that the BALB/c strain possesses a unique *Sirpa* polymorphism that enables the BALB/c SIRPA IgV domain to moderately bind human CD47. 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). Non-obese diabetes-resistant (NOR) mice homozygous for NOD-derived *Idd13* (NOR.NOD-*Idd13*) were purchased from 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 normal 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 (GM-CSF) (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 (APC)-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 FACS Aria 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 (Supplemental Table 1).

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 (HRP)-conjugated goat polyclonal antibody specific for the Fc γ fragment of human IgG (Jackson ImmunoResearch, Inc., 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. Non-linear regression analysis was performed to calculate K_d 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 (EGF) (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. Then, 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 RPMI1640 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 $1.5\text{-}3.5 \times 10^4$ human CB cells were opsonized with CD34 antibody (sc-19621; Santa Cruz, CA, USA), then added to the macrophages. Two hours after co-incubation 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 EIF α 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 kindly 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 by an FACS Aria (BD Biosciences) to obtain GFP⁺CD11b⁺ cells (Figure 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 2×10^2 – 2×10^3 cells per well, and then, 2×10^3 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 (CFCs) was counted after 2 weeks of cultures. LTC-ICs were defined as CFCs surviving after 4–5 weeks of stromal culture.

Statistical analysis

For the comparison of SIRPA-CD47 binding capabilities among strains, the K_d values in each group were estimated using the non-linear 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 non-linear 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 C57BL/6, NOD, and BALB/c strains on FACS. NOD macrophages bound strongly to human CD47-Fc protein, whereas C57BL/6 macrophages did not, confirming our previous results [16, 27]. Interestingly, BALB/c macrophages showed a low level binding capability to human CD47-Fc protein. Figure 1B showed the result of the SIRPA-CD47 binding assay to quantitate the affinity of SIRPA of each strain to human CD47-Fc protein. NOD SIRPA had strong affinity for human CD47 ($K_d = 2.501 \pm 0.274$, $B_{max} = 0.296 \pm 0.005$), and BALB/c SIRPA had an intermediate level of affinity ($K_d = 306.9 \pm 105.2$, $B_{max} = 0.156 \pm 0.028$), whereas C57BL/6 SIRPA did not bind to human CD47. These results are consistent with those of FACS analysis (Figure 1A).

It has been shown that the binding of cell-surface CD47 with SIRPA on macrophages activates tyrosine phosphatase SHP-1 to inhibit myosin assembly, preventing engulfment of macrophages [24, 25, 34, 35]. We then tested whether these differences in affinity for SIRPA reflects activation status of SHP-1 phosphorylation after exposure to human CD47-Fc protein (Figure 1C). The SHP-1 phosphorylation was not detected C57BL/6 macrophages. In contrast, a high level of SHP-1 (68 KDa) phosphorylation

was detected in NOD macrophages, and an intermediate level of SHP-1 phosphorylation was seen in BALB/c macrophages. These data suggest that BALB/c macrophages can activate “don’t eat me” signaling at an intermediate level, as a result of their modest binding capability to human CD47.

BALB/c-derived macrophages inhibit human LTC-ICs less effectively than C57BL/6-derived macrophages

We have shown that the capability of mouse bone marrow stromal cells to support human LTC-ICs reflects human cell engraftment *in vivo* in immunodeficient mouse models [16]. It is now clear that macrophages within the stromal cell layer play a critical role in inhibition of LTC-IC maintenance in this experiment. We have found that in long-term culture of human CB cells on MS-5 mouse stromal layer, the addition of C57BL/6 but not NOD macrophages strongly inhibited human LTC-IC maintenance. We therefore analyzed the effect of BALB/c macrophages on human LTC-IC maintenance utilizing this culture system. As shown in Figure 2A, when macrophages either from C57BL/6, BALB/c or NOD were added to the MS-5 stromal layer, this treatment suppressed human LTC-ICs in a dose-dependent manner. The addition of C57BL/6 macrophages was most effective for this suppression that of NOD macrophages was weak ($p < 0.01$), and that of BALB/c macrophages showed an intermediate effect ($p < 0.01$).

To confirm that this difference in inhibition of LTC-ICs is derived from strain-specific *Sirpa* polymorphisms, we cloned the *Sirpa* genes of

C57BL/6, NOD, and BALB/c strains, transduced each *Sirpa* lentivirally into C57BL/6 macrophages, and tested their ability to inhibit human LTC-ICs. As shown in Figure 2B, the enforcement of the NOD SIRPA expression in C57BL/6 macrophages strongly restored LTC-ICs, suggesting again that the affinity of SIRPA to human CD47 is a strong determinant of human LTC-IC maintenance in this assay system. Importantly, the enforced expression of BALB/c SIRPA in C57BL/6 macrophages modestly restored the LTC-IC maintenance, reasonably reflecting its intermediate affinity to human CD47.

Engulfment of human HSCs by macrophages is significantly inhibited in the BALB/c strain

Suppression of human LTC-IC maintenance by addition of mouse macrophages suggests that the target of engulfment should include early hematopoietic progenitor or stem cells. We have shown that in patients suffering from hemophagocytic lymphohistiocytosis (HLH), the CD34⁺CD38⁻ population that is enriched for human HSCs was the primary target of engulfment by activated macrophages to induce pancytopenia [26]. We thus tested whether the intermediate affinity of BALB/c SIRPA to human CD47 can prevent engulfment of human HSCs *in vitro*. Macrophages of each strain were cultured with human CD34⁺CD38⁻ cells, and the phagocytic index that represents the efficiency of engulfment by macrophages [26, 27, 32] was evaluated. As shown in Figure 3, C57BL/6 macrophages actively engulfed human HSCs, and showed the highest phagocytic index. In contrast, NOD macrophages showed the lowest value, and BALB/c macrophages showed an

intermediate value of phagocytic index. Collectively, “don’t eat me” signaling induced by a moderate binding of BALB/c SIRPA to human CD47 might prevent engulfment of HSCs by mouse macrophages, which might support LTC-IC maintenance (Figure 2).

The BALB/c mouse has *Sirpa* polymorphism within the IgV domain, which can affect the affinity to human CD47

The CD47 binding site on SIRPA is located in the distal extracellular IgV loop domain [25, 36, 37]. We analyzed the IgV domain amino acid sequence of mouse SIRPA to test whether the BALB/c mouse had polymorphisms of the *Sirpa* gene that might affect the binding affinity to human CD47. Critical residues of the *Sirpa* IgV domain for CD47 binding have been determined by multiple mutagenesis analyses in both of human and mouse [37-40]. X-ray crystallography analyses of human SIRPA-CD47 binding complex revealed that Val27 and Asp100 of human SIRPA IgV are critical for binding [37, 38, 41], which correspond to Leu29 and Asp104 in mouse SIRPA IgV, respectively (Figure 4).

The DNA sequence of *Sirpa* IgV domain was determined through PCR amplification of cDNA prepared from the peripheral blood of BALB/c, 129, ICR, and C3H mice. Results are listed in Figure 4, together with data of C57BL6, NOD, NOR *Sirpa* and human *SIRPA* that were published previously [16]. NOD and BALB/c had common polymorphisms such as Thr4, Val6 and Arg98, but our mutagenesis analysis revealed that these single nucleotide polymorphisms (SNPs) did not affect its affinity to human CD47

(unpublished data). BALB/c mice had two unique SNPs such as L29V, and substitution of Ile for Val at position 24 (V24I). Since Leu29 in mouse SIRPA corresponds to human Val27, the former substitution could enhance the affinity of mouse SIRPA to human CD47.

We then generated mutant C57BL/6 SIRPA carrying the L29V mutation, enforced to express this mutant in HeLa cells, and evaluated the affinity for human CD47-Fc protein. The affinity of mouse SIRPA to human CD47 was evaluated in the presence of 500 nM of human CD47. As shown in Figure 5A, HeLa cells expressing NOD SIRPA exhibited a strong affinity for human CD47, whereas C57BL/6 SIRPA did not. Cells expressing BALB/c SIRPA showed an intermediate level of affinity. Importantly, cells expressing C57BL/6 SIRPA with the L29V SNP also showed moderate affinity, whose level was comparable to that of cells expressing BALB/c SIRPA. These data strongly suggest that the L29V SNP is an element responsible to enhance the affinity of BALB/c SIRPA to human CD47.

In addition, we evaluated the effect of introduction of L29V SNP on the maintenance of human LTC-IC and the phagocytic activity against human hematopoietic cells, according to the methods used in Figures 2B and 3, respectively. One hundred C57BL/6 macrophages transduced lentivirally with SIRPA of C57BL/6, BALB/c or NOD strains, and with C57BL/6 SIRPA having the L29V SNP, were added to the MS-5 stromal layer. As shown in Figure 5B, by enforcing expression of the C57BL/6 SIRPA with L29V SNP, inhibition of C57BL/6 SIRPA expressing macrophages on human LTC-IC maintenance was significantly released, and became equivalent to that of

BALB/c SIRPA-expressing macrophages. The effect of introduction of L29V SNP on the phagocytic activity against human CD34⁺CD38⁻ stem and progenitor cells is shown in Figure 5C. Consistent with results of the LTC-IC assay (Figure 5B), macrophages expressing C57BL/6 SIRPA with the L29V SNP showed the phagocytic activity equivalent to that of macrophages expressing BALB/c SIRPA. These findings strongly suggest that BALB/c-specific L29V SNP contributed to the enhanced affinity of BALB/c SIRPA to human CD47, which might cause favorable xenograft efficiencies of human hematopoiesis in the BALB/c strain.

Discussion

Previous studies have shown that mouse strain is an important factor to establish human cell to mouse xenotransplantation systems: Like lymphoid-depleted immunodeficient mice of the NOD background, those of the BALB/c strain can support human hematopoietic reconstitution *in vivo*, although this strain effect is not as strong as the NOD strain does [7, 18]. We have shown that the efficient human cell engraftment in the immunodeficient NOD strain is attributable to the NOD-specific *Sirpa* polymorphism [27].

In the present paper, we show that the polymorphism of the BALB/c *Sirpa* might also be critical to prevent macrophage-mediated suppression of human hematopoiesis. Although a previous report showed that the binding affinity of BALB/c SIRPA for human CD47 was almost absent in the presence of 60nM human CD47-Fc protein[42], we found that BALB/c SIRPA showed significant binding affinity at higher concentrations of human CD47-Fc protein (Figure 1B). The BALB/c-specific polymorphic SIRPA had an affinity for human CD47 at an intermediate level, provoking inhibitory signals such as SHP-1 phosphorylation for macrophages to engulf early hematopoietic stem or progenitor cells, which allowed maintenance of LTC-ICs by MS-5 stromal cells in the presence of BALB/c macrophages. The enforced expression of polymorphic BALB/c SIRPA in C57BL/6 macrophages rendered them not to inhibit LTC-ICs, and this effect was explainable at least by the L29V SNP of the BALB/c SIRPA. These data strongly suggest that the affinity of strain-specific SIRPA to human CD47 is a decisive factor

for efficiency of xenotransplantation.

Interestingly, the degree of the affinity of strain-specific SIRPA appears to correlate with the efficiency of strain-specific transplantation capability. In parallel with an intermediate affinity of BALB/c SIRPA to human CD47, the SHP-1 protein was moderately phosphorylated in BALB/c macrophages, as compared to the level of SHP-1 phosphorylation in NOD macrophages in response to human CD47 (Figure 1C). It has been reported that human neoplastic cells or leukemic stem cells express higher levels of CD47 than normal cells do, rendering malignant cells to be able to escape from engulfment by macrophages, which endows malignant cells with growth advantages over normal cells [43]. Therefore, in developing efficient xenotransplantation models, further modification of mouse SIRPA to enhance binding to human CD47 should be critical to minimize the inhibitory effect of host macrophages.

It is also important to understand the target cell for engulfment in the xenogeneic transplantation setting. We have reported that disruption of the SIRPA-CD47 binding primarily induces development of human HLH [26]. In HLH, the human CD34⁺CD38⁻ HSC population downregulates CD47 in response to hypercytokinemia, causing HSCs to be engulfed by macrophages. Interestingly, also in the xenogeneic transplantation setting, we showed that the target of engulfment includes the HSC population (Figure 3). Furthermore, macrophages from the BALB/c mouse engulfed human CD34⁺CD38⁻ cells at an intermediate level between those seen in the C57BL/6 and in the NOD mouse (Figure 3), corresponding well to the results

of LTC-IC assays (Figure 2). Thus, the strain-specific inefficiencies for suppression of mouse macrophages should directly relate to the severity of rejection of human HSCs in xenogeneic transplantation models.

The CD47 binding site on SIRPA is located in the IgV loop domain [25, 36, 37]. The extracellular IgV domain is relatively well conserved (>75%) in both mouse and human SIRPA [37], but the binding to CD47 is species-specific. It is probable that the residues at the binding site in the loop domain have a great effect on the shape of the loop by small chemical differences in side chains, resulting in the acquisition of diverse ligand-binding specificities [37, 38]. We identified 2 SNPs unique to BALB/c mice, and 3 SNPs unique to both BALB/c and NOD mice (Figure 4). Among these 5 SNPs, the 29th amino acid in mouse SIRPA appeared to be critical because it corresponds to the 27th amino acid in human SIRPA that was critical for human CD47 binding [38-40]. Interestingly, BALB/c and human SIRPA have a Val residue at these sites, whereas all other mouse strains have a Leu residue (Figure 4). Introduction of C57BL/6 SIRPA carrying the L29V mutation into C57BL/6 macrophages conferred the binding affinity to human CD47 equivalent to that of BALB/c SIRPA, resulting in partial release of human LTC-IC inhibition and phagocytosis against human CD34⁺CD38⁻ cells (Figure 5). These results strongly suggest that the L29V mutation contributes to the enhanced binding of BALB/c SIRPA to human CD47, and therefore to higher xenograft efficiencies in BALB/c strain as compared to those in the conventional C57BL/6 strain. Because Val has a slightly shorter hydrophobic side-chain, this exchange may alter

conformation of the loop to increase the binding affinity of mouse SIRPA to human CD47.

Conclusion

BALB/c mice have the strain-specific L29V polymorphism in SIRPA, which renders it to be able to recognize human CD47. Our data strongly suggest that one of the critical determinants for strain-specific efficiency of xenogeneic transplantation might be the affinity of the SIRPA-CD47 binding that is decided by strain-specific SIRPA polymorphisms. This information is useful to establish a novel, more efficient immunodeficient mouse models for human cell transplantation.

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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Figure legends

Figure 1. Binding capability of mouse SIRPA to human CD47

(A) Binding of the human CD47-Fc fusion protein to SIRPA-expressing macrophages derived from C57BL/6, NOD, and BALB/c mice by flow cytometry analysis.

(B) Dose-response curves for the mouse SIRPA-human CD47-Fc protein interaction determined by SIRPA-CD47 binding assay. Human CD47-Fc protein binding to mouse SIRPA was detected using peroxidase-conjugated goat anti-human Fc antibody. A peroxidase activity was evaluated by the absorbance at 490nm. $**p < 0.01$. Results shown are representative of four independent experiments.

(C) Comparison of the amount of phosphorylated SHP-1 in macrophages after exposure to human CD47-Fc protein, as determined by immunoblot analysis.

Figure 2. SIRPA modulates mouse macrophage-mediated suppression of human hematopoiesis

(A) Inhibition of human LTC-ICs on the MS-5 mouse stromal layer by the addition of macrophages derived from each strain.

(B) Effects of macrophages with enforced strain-specific SIRPAs on human LTC-IC maintenance. A schematic illustration of the experimental design is shown at the upper panel. $**p < 0.01$. Each experiment was done with five replicates per dose. Results shown are representative of three

independent experiments.

Figure 3. Phagocytosis of human CD34⁺CD38⁻ hematopoietic stem/progenitor cells by mouse macrophages

The phagocytic index was determined as the number of engulfed cells per 100 macrophages. Bars indicate mean \pm SD. * $p < 0.05$. ** $p < 0.01$.

Figure 4. DNA sequencing of the mouse *Sirpa* and human *SIRPA* IgV domain

SNPs specific only to the BALB/c strain, and those to both NOD and BALB/c strains are boxed in orange. Sequences of the human *SIRPA* IgV domain are aligned to those of the mouse *Sirpa* IgV domain. The residues of human *SIRPA* critical to bind human CD47 are boxed in green.

Figure 5. BALB/c-specific SNP L29V confers affinity for human CD47

(A) Human CD47-Fc protein binding to HeLa cells infected with C57BL/6, NOD, BALB/c *SIRPA*, and C57BL/6 *SIRPA* constructs carrying the L29V mutation was evaluated in the presence of 500nM of human CD47 by a peroxidase activity assay. Binding to human CD47-Fc protein is expressed as a peroxidase activity determined by the absorbance at 490nm. ** $p < 0.01$.

The results shown are representative of three independent experiments.

(B) Effects of macrophages with enforced SNP L29V *SIRPA* on human LTC-IC maintenance. C57BL/6 macrophages expressing strain-specific and

SNP L29V SIRPA was added on the MS-5 stromal layer. Each experiment was done with five replicates at a dose of 100 mouse macrophages per well. Results shown are representative of three independent experiments. * $p < 0.05$. ** $p < 0.01$.

(C) Phagocytosis of human CD34⁺CD38⁻ hematopoietic stem/progenitor cells by mouse macrophages with enforced SNP L29V SIRPA. The phagocytic index was determined as the number of engulfed cells per 100 macrophages. Bars indicate mean \pm SD. * $p < 0.05$.

Supplemental Table 1. Primer sets to examine the sequences of mouse *Sirpa* CDS and *Sirpa* IgV domains