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, 2×10^3 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

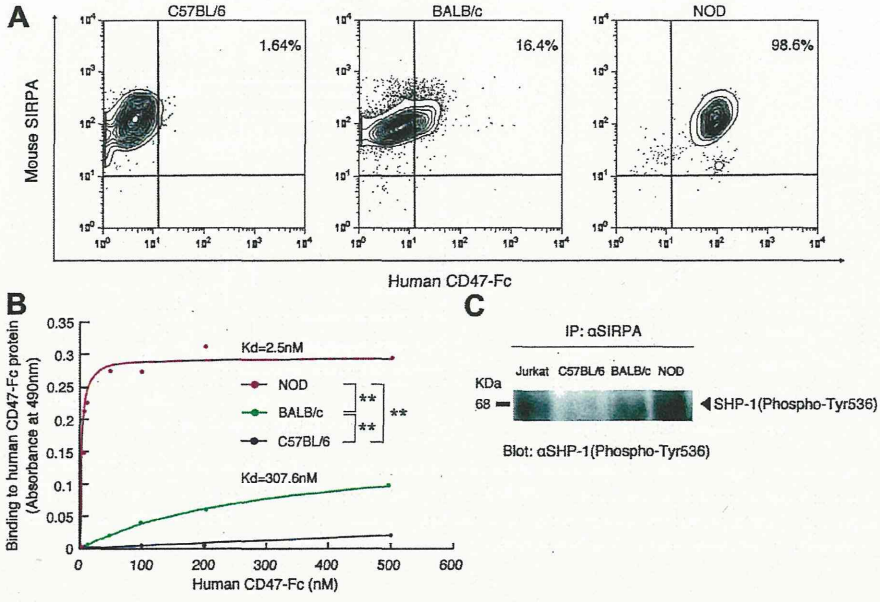

Figure 1. Binding capability of mouse SIRPA to human CD47. (**A**) Binding of the human CD47-Fc fusion protein to SIRPA-expressing macrophages derived from C57BL/6, NOD, and BALB/c mice by flow cytometry analysis. (**B**) Dose-response curves for the mouse SIRPA-human CD47-Fc protein interaction determined 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 490 nm. $^{**}p < 0.01$. 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.

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 (Kd = 2.501 ± 0.274; Bmax = 0.296 ± 0.005), and BALB/c SIRPA had an intermediate level of affinity (Kd = 306.9 ± 105.2; Bmax = 0.156 ± 0.028), whereas C57BL/6 SIRPA did not bind to human CD47. These results are consistent with those of FACS analysis (Fig. 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 (Fig. 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 because 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 using this culture system. As shown in Figure 2A, when macrophages 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

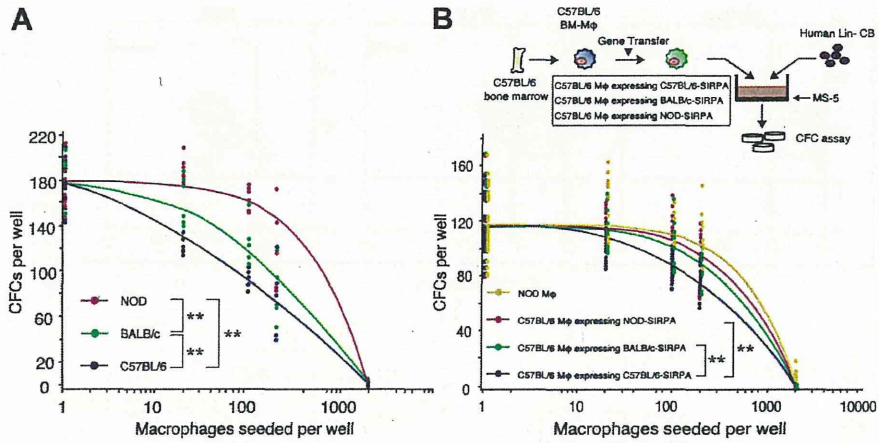

Figure 2. SIRPA modulates mouse macrophage-mediated suppression of human hematopoiesis. (**A**) Inhibition of human LTC-ICs on the MS-5 mouse stromal layer by the addition of macrophages derived from each strain. (**B**) Effects of macrophages with enforced strain-specific SIRPAs on human LTC-IC maintenance. 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.

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 the 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 (Fig. 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 humans and mice [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 (Fig. 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 (C. Iwamoto, unpublished data). BALB/c mice had two unique SNPs such as L29V, and substitution of Ile for Val at position 24 (V24I). Because 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

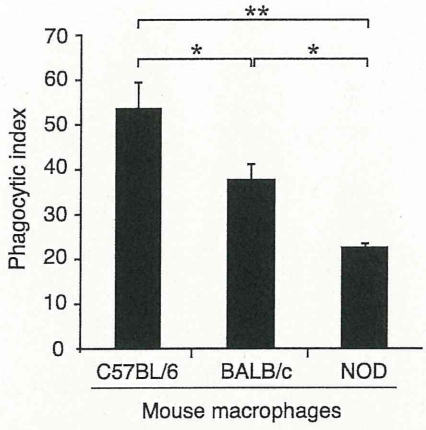

Figure 3. Phagocytosis of human $CD34^+CD38^-$ hematopoietic stem/progenitor cells by mouse macrophages. The phagocytic index was determined as the number of engulfed cells per 100 macrophages. Bars indicate mean ± SD. $*p < 0.05$; $**p < 0.01$.

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 stains, 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 (Fig. 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 this study, 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 60 nM of human CD47-Fc protein [42], we found that BALB/c SIRPA showed significant binding affinity at higher concentrations of human CD47-Fc protein (Fig. 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

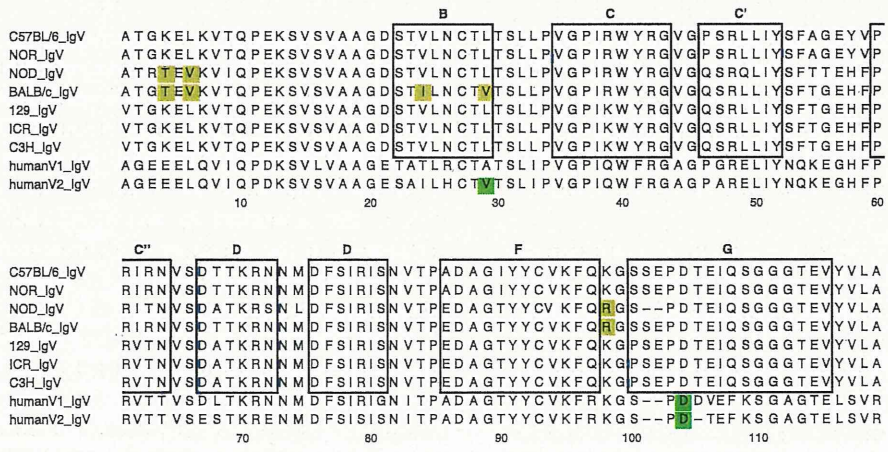

Figure 4. DNA sequencing of the mouse *Sirpa* and human *SIRPA* IgV domain. SNPs specific only to the BALB/c strain, and those to both NOD and BALB/c strains, are boxed in orange. Sequences of the human *SIRPA* IgV domain are aligned to those of the mouse *Sirpa* IgV domain. The residues of human SIRPA critical to bind human CD47 are boxed in green.

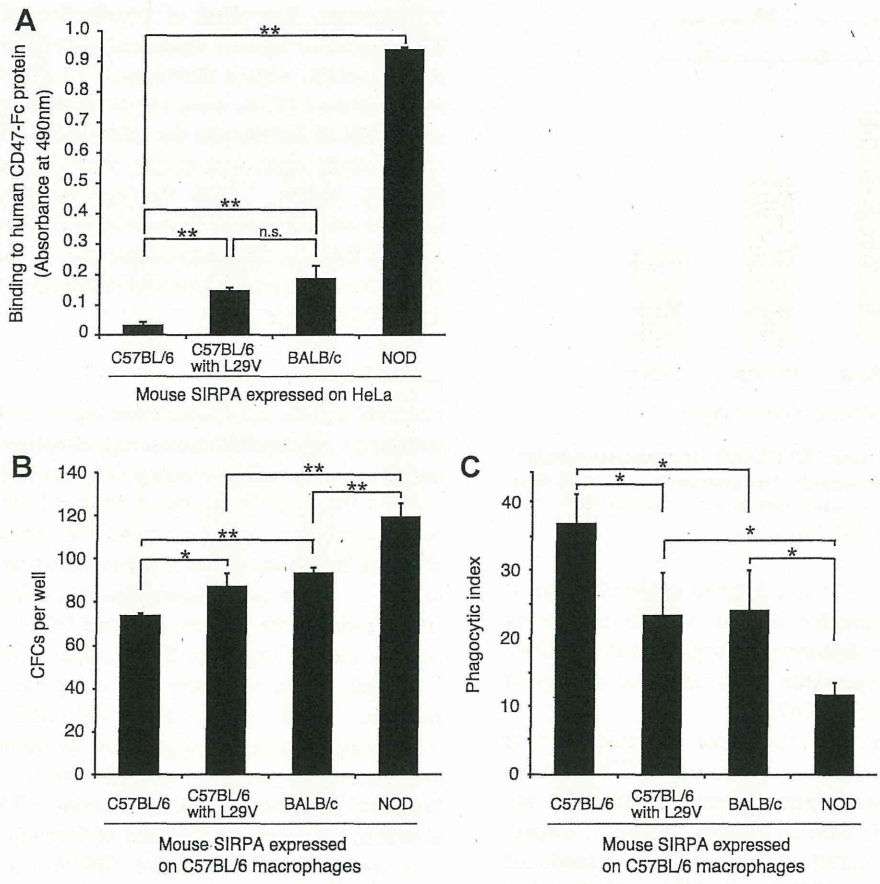

Figure 5. BALB/c-specific SNP L29V confers affinity for human CD47. (**A**) Human CD47-Fc protein binding to HeLa cells infected with C57BL/6, NOD, BALB/c SIRPA, and C57BL6 SIRPA constructs carrying the L29V mutation was evaluated in the presence of 500 nM of human CD47 by a peroxidase activity assay. Binding to human CD47-Fc protein is expressed as a peroxidase activity determined by the absorbance at 490 nm. 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. (**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 ± SD. $*p < 0.05$; $**p < 0.01$.

macrophages. The enforced expression of polymorphic BALB/c SIRPA in C57BL/6 macrophages rendered them unable 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 with the level of SHP-1 phosphorylation in NOD macrophages in response to human CD47 (Fig. 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 (Fig. 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 (Fig. 3), corresponding well to the results of LTC-IC assays (Fig. 2). Thus, the strain-specific inefficiencies for suppression of mouse macrophages should relate directly 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 two SNPs unique to BALB/c mice and three SNPs unique to both BALB/c and NOD mice (Fig. 4). Among these five 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 (Fig. 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 (Fig. 5). These results strongly suggest that the L29V mutation contributes to the enhanced binding of BALB/c SIRPA to human CD47 and thus to higher xenograft efficiencies in BALB/c strain, as compared with those in the conventional C57BL/6 strain. Because Val has a slightly shorter hydrophobic side-chain, this exchange could 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.

Acknowledgments

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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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Supplementary Table E1. Primer sets to examine the sequences of mouse *Sirpa* CDS and *Sirpa* IgV domains

Name	Primer sequence
C57BL/6 cDNA Sirpa 1F	5′-ATGGAGCCCGCCGGCCCGGCCCCTGGC-3′
C57BL/6 cDNA Sirpa 1R	5′-TCACTTCCTCTGGACCTGGACACTAGC-3′
129 cDNA Exon4 1F	5′-GAGTCACGGGGAAAGAACTG-3′
Exon4R	5′-CGAGTACATAGACCTCTGT-3′
C57BL/6 cDNA Exon3 1F	5′-GCTCTCCGCGTCCTGTTTCTGTACAG-3′
C57BL/6 cDNA Exon5 1R	5′-GGGAGAGAAGCCATGAGACTTGCAGG-3′

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Brief Communication

GATA2 zinc finger 2 mutation found in acute myeloid leukemia impairs myeloid differentiation

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ABSTRACT

We identified two novel *GATA2* mutations in acute myeloid leukemia (AML). One mutation (p.R308P-GATA2) was a R308P substitution within the zinc finger (ZF)-1 domain, and the other (p.A350_N351ins8-GATA2) was an eight-amino-acid insertion between A350 and N351 residues within the ZF-2 domain. p.R308P-GATA2 did not affect DNA-binding and transcriptional activities, while p.A350_N351ins8-GATA2 reduced them, and impaired G-CSF-induced granulocytic differentiation of 32D cells. Although p.A350_N351ins8-GATA2 did not show a dominant-negative effect over wild-type (Wt)-GATA2 by the reporter assay, it might be involved in the pathophysiology of AML by impairing myeloid differentiation because of little Wt-GATA2 expression in primary AML cells harboring the p.A350_N351ins8 mutation.

1. Introduction

GATA transcription factors contribute to the regulation of cell lineage commitment and differentiation.[1] Although hematopoiesis is controlled by numerous transcription and signaling factors with tightly integrated functions, GATA1, GATA2 and GATA3 in the GATA family are involved in the developmental regulation of hematopoiesis. In addition to the essential role in normal hematopoiesis, recent studies demonstrated that mutation of *GATA* genes is involved in the development and progression of leukemia. p.L359V mutation of *GATA2* gene was identified in about 10% of chronic myeloid leukemia (CML) cases at accelerated phase and myeloid blast crisis.[2] Moreover, recent studies demonstrated that *GATA2* gene mutations were identified in AML patients and in three different familial syndromes characterized by predisposition to myelodysplastic syndrome (MDS) and AML.[3–5] These results collectively indicate that dysregulation of GATA2 might be involved in the development and/or progression of AML.

In this study, we analyzed the biological effects of two novel *GATA2* mutations, which were identified in adult *de novo* AML patients.

2. Materials and methods

The diagnosis of AML was based on the morphology, histopathology, the expression of leukocyte differentiation antigens and/or the French–American–British (FAB) classification. *GATA2* mutation was screened in 96 newly diagnosed *de novo* AML adult patients. Informed consent was obtained from all patients to use their samples for banking and molecular analysis, and approval was obtained from the ethics committee of Nagoya University School of Medicine.

The full-length human wild-type (Wt)- and mutated (Mt)-GATA2 cDNAs were amplified from Wt- or Mt-GATA2-expressing leukemia cells, respectively. C-terminally Myc-tagged GATA2 cDNAs were cloned into pcDNA3.1 vector, and electrophoretic mobility shift assay (EMSA) and luciferase assay were performed as previously reported.[6]

C-terminally Myc-tagged Wt- and mutant-GATA2 cDNAs cloned in the pMX-IP vector were transduced into murine IL3-dependent myeloid progenitor cell line 32D cells as previously described.[7] Stable Wt- and mutant-GATA2-expressing 32D cells were subjected to immunofluorescence, proliferation and differentiation analyses.

Wt- and mutant-GATA2-expressed 32D cells were suspended in RPMI1640 medium containing 10% FCS with an increasing concentration of murine IL3 (R&D Systems), and 2×10^4 cells per well were seeded in 96-well culture plates. Cell viability was measured using the CellTiter96 Proliferation Assay (Promega). For the induction of myeloid differentiation, Wt- and mutant-GATA2-expressed 32D cells were cultured in RPMI1640 medium containing 10% FCS and 30 ng/ml recombinant G-CSF (Kyowa-Kirin, Tokyo, Japan) without IL3.

Luciferase assay and cell proliferation and differentiation analyses were performed three times independently. Differences in continuous variables were analyzed with unpaired *t* test for the

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