TRANSPLANTATION

Donor SIRPα polymorphism modulates the innate immune response to allogeneic grafts

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Mice devoid of T, B, and natural killer (NK) cells distinguish between self and allogeneic nonself despite the absence of an adaptive immune system. When challenged with an allograft, they mount an innate response characterized by accumulation of mature, monocyte-derived dendritic cells (DCs) that produce interleukin-12 and present antigen to T cells. However, the molecular mechanisms by which the innate immune system detects allogeneic nonself to generate these DCs are not known. To address this question, we studied the innate response of *Rag2*$^{-/-}\gamma c^{-/-}$ mice, which lack T, B, and NK cells, to grafts from allogeneic donors. By positional cloning, we identified that donor polymorphism in the gene encoding signal regulatory protein α (SIRPα) is a key modulator of the recipient's innate allorecognition response. Donors that differed from the recipient in one or both *Sirpa* alleles elicited an innate alloresponse. The response was mediated by binding of donor SIRPα to recipient CD47 and was modulated by the strength of the SIRPα-CD47 interaction. Therefore, sensing SIRPα polymorphism by CD47 provides a molecular mechanism by which the innate immune system distinguishes between self and allogeneic nonself independently of T, B, and NK cells.

INTRODUCTION

Recognition of allogeneic nonself by the mammalian immune system has been attributed to adaptive lymphoid cells that express rearranging receptors for antigen (T and B lymphocytes) or to natural killer (NK) cells that express nonrearranging receptors but are activated in response to "missing self" (*1*, *2*). The principal alloantigens recognized by lymphocytes and NK cells are the polymorphic major histocompatibility complex (MHC) molecules widely expressed on bodily tissues. Transplantation of tissues between MHC-mismatched individuals therefore triggers a potent lymphoid cell response that causes graft rejection.

Recent studies have shown that innate myeloid cells, which have elaborate systems for sensing microbial nonself, also engage in allorecognition (*3*). Upon encountering nonself cells or tissues transplanted from another individual, monocytes cause a delayed-type hypersensitivity–like reaction or differentiate into potent antigen-presenting dendritic cells (DCs) independently of T, B, and NK cells (*4*–*6*). In one model (*5*), heart allografts transplanted to *Rag2*$^{-/-}\gamma c^{-/-}$ mice, which lack all lymphoid cells, were persistently infiltrated with mature monocyte-derived DCs (mono-DCs) that expressed interleukin-12 (IL-12) and stimulated both T cell proliferation and interferon-γ (IFN-γ) production (*5*). In contrast, syngeneic grafts in the same mice harbored a smaller number of mono-DCs transiently, and these DCs neither expressed IL-12 nor stimulated IFN-γ production by T cells. Only T cells activated by mono-DCs that were generated in response to allogeneic nonself caused graft rejection (*5*). Furthermore, mono-DCs that accumulated in the graft directly contributed to rejection by propagating the local effector T cell response (*7*). Other studies have shown that macrophages also respond to allogeneic nonself. Macrophages from mice primed with allogeneic cells subsequently killed cells grafted from the same donor, although this allotoxic response depended on $CD4^+$ T lymphocyte help at the time of priming (*8*). Therefore, nonself sensing by innate myeloid cells is not restricted to microbes but extends to the recognition of allografts.

How monocytes or macrophages distinguish between self and allogeneic nonself is not known. Earlier studies suggested that activation of monocytes by allogeneic grafts is not dependent on MHC mismatch between donor and recipient but rather on mismatches elsewhere in the genome (*4*–*6*). This raised the possibility that genetically defined non-MHC determinants on donor cells control the host's innate alloresponse. Identifying these determinants would provide fundamental insights into innate immune recognition mechanisms central to the transplant rejection and the maternal immune response to the allogeneic fetus. We therefore embarked on a genetic mapping (positional cloning) study in the mouse that exploited differences in the magnitude of the monocyte response elicited by allografts from congenic inbred donors. We report here that a key determinant of the innate immune response to allogeneic nonself is donor polymorphism in the immunoglobulin superfamily (IgSF) membrane protein SIRPα that is sensed by CD47 on recipient monocytes.

RESULTS

Magnitude of the host innate alloresponse is influenced by the genetic background of the donor

To explore the mechanisms by which the innate immune system senses allogeneic grafts, we tested the innate response of lymphoid cell–deficient mice to grafts from genetically disparate donors. Bone

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marrow plugs from six common laboratory [DBA/2, nonobese diabetes-resistant (NOR), C3H, FVB, BALB/c, and nonobese diabetic (NOD)] and three wild-derived [*Mus Pahari*, Watkins Star Line B (WSB), and CAST] inbred mouse strains were transplanted individually under the kidney capsules of separate C57BL/B6 (B6) *Rag2*$^{-/-}\gamma c^{-/-}$ (BRG) recipients. BRG mice are devoid of lymphoid cell lineages and lack known T, B, and NK cell–mediated allorecognition. Control BRG mice were transplanted with bone marrow plugs from syngeneic B6 donors that were wild-type at the *Rag2* and γ*c* loci. Plugs were removed 1 week after transplantation, and the number of infiltrating host mono-DCs was quantified by flow cytometry as a measure of an innate alloresponse (fig. S1) (*5*). As shown in Fig. 1A, allogeneic grafts harbored significantly greater numbers of host mono-DCs than did syngeneic B6 grafts, with the largest mono-DC infiltrate observed in NOD allografts. In contrast, mono-DC infiltration of syngeneic NOD grafts transplanted to NOD.*Rag2*$^{-/-}\gamma c^{-/-}$ (NRG) recipients was minimal (Fig. 1B), indicating that the robust mono-DC infiltrate observed in NOD allografts is a true measure of an innate alloresponse. We also tested the host response to allogeneic (NOR, BALB/c, or NOD) and syngeneic (B6) bone marrow plugs transplanted simultaneously in the contralateral kidneys of the same BRG recipient. NOR mice are a recombinant inbred strain that is 88% identical by descent to NOD, including at the *Mhc* locus (*9*, *10*). Allogeneic BALB/c, but not allogeneic NOR grafts, elicited greater mono-DC infiltration than syngeneic B6 grafts (Fig. 1C). In addition, NOD grafts displayed a much larger mono-DC infiltrate than did either B6 or NOR grafts (Fig. 1C), confirming that NOD donor tissue induces a robust innate alloresponse. The difference between NOD and NOR allografts was also observed in BALB/c.*Rag2*$^{-/-}\gamma c^{-/-}$ (CRG) recipients (Fig. 1D). Therefore, varying the genetic background of the donor modulates the host innate alloresponse, with NOD allografts inducing the strongest response, whereas allografts from the closely related NOR mouse inducing a weak response.

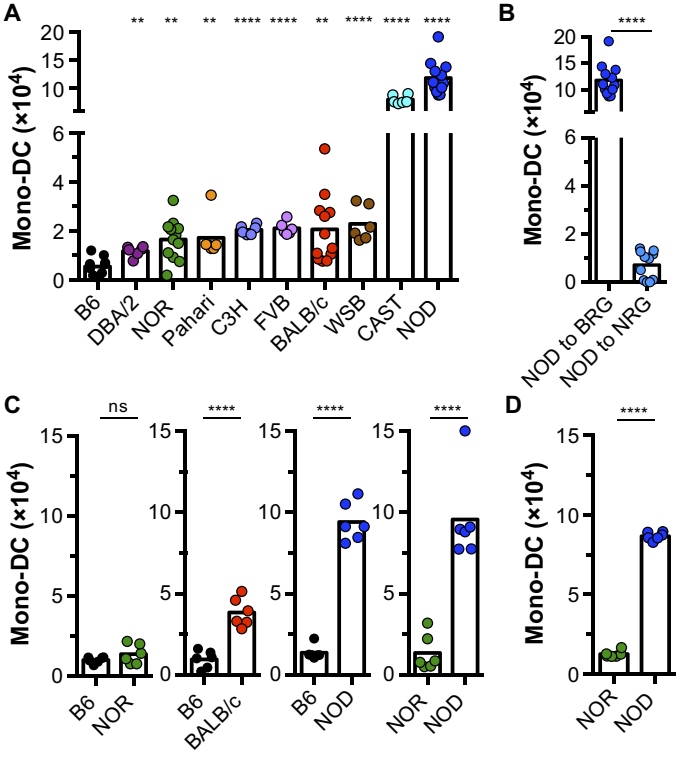

Fig. 1. Magnitude of the host innate alloresponse is influenced by the genetic background of the donor. Bone marrow plugs from different donors were transplanted individually under the kidney capsules of separate mice (**A**) or simultaneously in the contralateral kidneys of the same mouse (**B** and **C**). All recipients were BRG unless otherwise stated. Donor strains are shown on the *x* axis. The number of recipient mono-DC infiltrating the grafts was determined 1 week later as a measure of an innate alloresponse. (A) Responses of BRG recipients to allografts from six common (DBA.2, NOR, C3H, FVB, BALB/c, and NOD) and three wild-derived (Pahari, WSB, and CAST) inbred strains. Statistical significance shown is relative to B6. (B) NOD grafts transplanted to syngeneic NRG mice (NOD to NRG) elicit a much weaker response than NOD grafts transplanted to allogeneic BRG mice (NOD to BRG). (C and **D**) Innate responses elicited by grafts from distinct donors transplanted under the contralateral kidney capsules of the same BRG recipient (C) or CRG recipient (D). $n = 5$ to 6 mice per group per experiment. Experiments were performed once or twice. Each dot represents an individual biological replicate. Bars are means. $^{**}P < 0.01$, $^{****}P < 0.0001$; ns, not significant (unpaired two-tailed *t* test).

Innate alloresponse is determined by a single Mendelian locus in the donor not linked to the *Mhc*

To determine whether donor variables that influence the magnitude of the innate alloresponse are heritable, we crossed BRG and NRG mice and studied the response induced by F_1 and F_2 grafts transplanted to BRG recipients. As shown in Fig. 2A, grafts from F_1 progeny induced an intermediate response (they were infiltrated with about half the number of host mono-DCs as NRG grafts), whereas responses elicited by F_2 grafts segregated at a ~1:2:1 (7:15:8) ratio into weak (equivalent to BRG grafts), intermediate (equivalent to F_1 grafts), and strong (equivalent to NRG grafts), respectively. These outcomes are consistent with the inheritance of a single Mendelian locus at which alleles exhibit an additive effect. Because all mice used in these experiments were on the *Rag2*$^{-/-}\gamma c^{-/-}$ background, the results also confirmed our previous demonstration that the alloresponse is not dependent on lymphoid cells in either the donor or the recipient (*5*).

The fact that NOR and NOD grafts elicited distinctly different responses (Fig. 1), despite sharing the same *Mhc* locus (*9*, *10*), suggested that the locus controlling the magnitude of the alloresponse is not linked to the *Mhc*. This is supported by experiments in which we transplanted bone marrow plugs from BALB.B (H-2^b) donors into BRG (H-2^b) recipients, a donor-host combination that is genetically identical at the *Mhc* but disparate at non-*Mhc* loci. BALB.B grafts exhibited significant mono-DC infiltration (Fig. 2B), comparable in magnitude to BALB/c grafts (Fig. 1, A and B). Conversely, grafts from B6.C mice, which carry a distinct *Mhc* haplotype (H-2^d) but are identical at all other loci to BRG recipients, elicited a weaker response than did BALB.B grafts (Fig. 2B), comparable with that of B6 grafts (Fig. 1). Therefore, mismatches at non-*Mhc* loci between donor and recipient are necessary and sufficient to trigger the innate alloresponse. We also observed that the alloresponse was not diminished when bone marrow plugs were derived from MHC class I (MHC I) (β_2-microglobulin)–deficient (NOD.*Prkdc*^{scid}.*b2m*$^{-/-}$) mice (Fig. 2C) compared with grafts from similarly lymphoid-deficient but MHC I–sufficient NRG mice (Fig. 2A). Similarly, MHC II expression was not required on the allografts because B6.*Mhc2*$^{-/-}$ and parental B6 allografts had equivalent mono-DC infiltration in CRG recipients (Fig. 2D). Thus, a single non-*Mhc* locus in the donor controls the magnitude of the innate alloresponse, and neither MHC disparity between donor and recipient nor expression of MHC I or MHC II molecules on graft is necessary for eliciting the response.

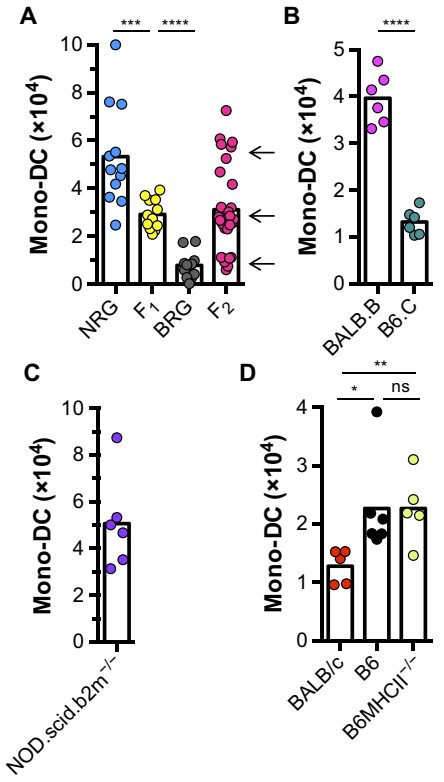

Fig. 2. Innate alloresponse is determined by a single Mendelian locus in the donor not linked to the *Mhc*. Bone marrow plugs were transplanted individually under the kidney capsules of separate mice. All recipients were BRG except in (**D**) where they were CRG. Donor strains are shown on the *x* axis. Recipient mono-DCs in grafts were measured as in Fig.1. (**A**) Responses of BRG recipients to grafts from parental NRG and BRG strains or to grafts from (BRG × NRG)F_1 and F_2 generations. All donors and recipients were on the *Rag2*$^{-/-}$γc$^{-/-}$ background. (**B**) Effect of donor-recipient non-MHC mismatch (BALB.B grafts) or MHC mismatch (B6.C grafts) on the innate alloresponse of BRG recipients. (**C**) Effects of donor MHC I deficiency (NOD.scid.b2m$^{-/-}$ grafts) on the innate alloresponse of BRG recipients. (D) Effect of MHC II deficiency (B6.MHCII$^{-/-}$ grafts) on the innate alloresponse of CRG recipients. n = 5 to 6 mice per group per experiment, except in F_2 experiment (n = 30 mice transplanted in two separate batches). Experiments were performed once or twice. Each dot represents an individual biological replicate. Bars are means. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (unpaired two-tailed t test).

Innate alloresponse maps to a small genomic region in the donor containing the *Sirpa* gene

The conspicuous difference in mono-DC infiltration between NOD and NOR grafts (Fig. 1) provided the opportunity for fine genetic mapping of the innate alloresponse locus in the donor because of the availability of well-characterized NOD.NOR congenic mouse lines. NOD and NOR congenic mouse strains share about 88% genetic identity but differ in their susceptibilities to autoimmune diabetes. Subcongenic lines generated by introgressing genetic intervals from NOR to NOD mice have enabled the mapping of important diabetes susceptibility and immune response traits in the NOD mouse (*10*–*13*). One example is the superiority of the immunodeficient NOD.*Prkdc*^{scid} mouse as host for human hematopoietic stem cells compared with equally immunodeficient NOR or B6 recipients. Genetic control of this trait was due to polymorphism at the gene encoding SIRPα in the recipient (*14*, *15*). SIRPα is an IgSF receptor expressed on myeloid cells, neurons, and other cell types (*16*, *17*). SIRPα engagement by its ubiquitously expressed monomorphic ligand CD47 delivers an inhibitory signal that represses phagocytosis by macrophages and inhibits multiple aspects of DC activation (*18*–*20*). In some contexts, CD47-SIRPα signaling is bidirectional because SIRPα binding to CD47 triggers stimulatory signals in T cells and neurons (*12*, *21*, *22*). We therefore took a genetic approach to test whether donor SIRPα polymorphism controls the MHC-independent alloreactions we had observed. We used a series of congenic NOD strains carrying different NOR-derived genomic intervals of chromosome 2 that did or did not contain *Sirpa* as bone marrow donors for BRG recipients (*12*, *14*). Bone marrow plugs from NOD.*NOR-Ila-D2Gul482* (NOD.*NOR-R7*; referred to as R7) donors, which are identical to the parental NOD strain except for a ~2-megabyte segment that includes NOR-*Sirpa* (Fig. 3A), elicited an alloresponse indistinguishable from parental NOR grafts (Fig. 3B). In contrast, bone marrow from NOD.*NOR-D2Gul169-D2Gul289* (NOD.*NOR-R12*; referred to as R12) mice that differ from the parental NOD strain in a different genomic segment compared with R7 and express the NOD-derived SIRPα (Fig. 3A) phenocopied parental NOD donors (Fig. 3B). This result was confirmed by showing that NOD and R12 bone marrow plugs transplanted into contralateral kidneys of the same BRG recipient displayed equivalent levels of mono-DC infiltration (Fig. 3C). Last, we transplanted grafts from a BRG donor strain that is congenic for the NOD-derived *Sirpa* allele (BRGS) (*15*) and observed that these bone marrow plugs stimulated an innate alloresponse equivalent to NRG donor tissue (Fig. 3D). Therefore, the capacity of donor tissue to induce a robust innate alloresponse, characterized by mono-DC infiltration of the graft, mapped to a very small region in the mouse genome that encodes the polymorphic *Sirpa* gene.

Donor SIRPα binding to recipient CD47 is required for triggering the innate alloresponse

To determine whether the binding of SIRPα on donor tissue to its ligand CD47 on recipient cells is necessary to elicit the innate alloresponse, we treated BRG recipients of NOD grafts with human CD47-Fc (hCD47-Fc), a fusion protein that binds NOD SIRPα on graft cells with high affinity and prevents it from binding to its native ligand mouse CD47 (*14*). Compared with isotype control human IgG1 Fc (hIgG1 Fc) protein, hCD47-Fc treatment inhibited the accumulation of mono-DCs in NOD grafts (Fig. 4A). Moreover, BRG recipients carrying a targeted deficiency in *Cd47* (BRG.*Cd47*$^{-/-}$) (*23*) did not respond to either NOD or BALB/c grafts (Fig. 4B). These data establish that SIRPα expressed on donor cells activates the host's innate alloresponse by engaging CD47.

We considered the possible confounder that donor SIRPα binding to CD47 on recipient mono-DCs could inhibit the phagocytic function of donor myeloid cells in the bone marrow graft, indirectly influencing the number of infiltrating mono-DCs. To test this possibility, we transplanted bone marrow plugs from B6.*Sirpa*^{tm1} mice with a targeted deletion of the cytoplasmic region of SIRPα required for signaling (*24*). Allogeneic B6.*Sirpa*^{tm1} bone marrow plugs transplanted to CRG recipients accumulated the same number of host mono-DCs as parental B6 grafts that express a full-length SIRPα protein (Fig. 4C). This could not be explained by inherent hyperactivity of cells in B6.*Sirpa*^{tm1} bone marrow grafts because increased host mono-DC accumulation was not observed when these grafts were transplanted to syngeneic BRG mice (Fig. 4D). Therefore, eliminating

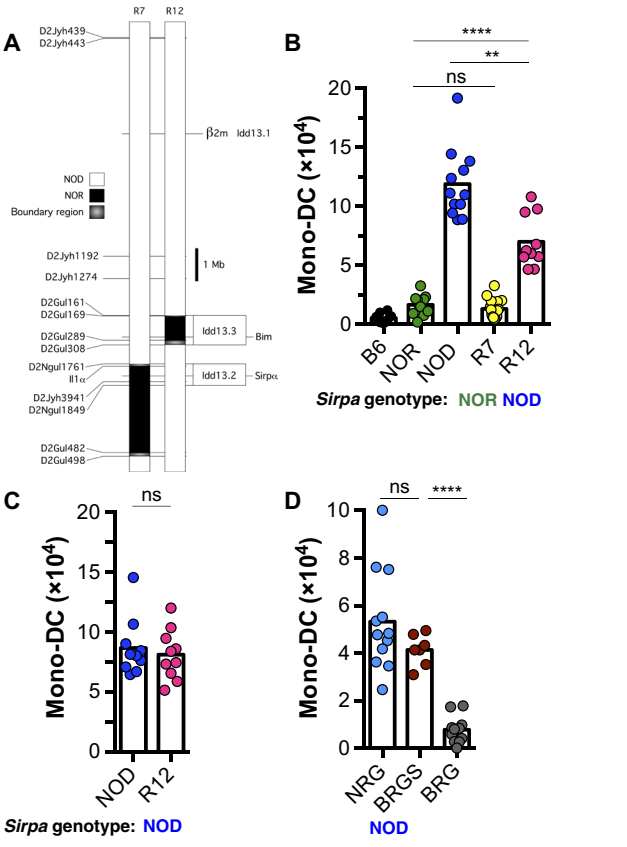

Fig. 3. Innate alloresponse maps to a small genomic region in the donor containing the *Sirpa* gene. Bone marrow plugs were transplanted individually under the kidney capsules of separate mice (**B** and **D**) or simultaneously in the contralateral kidneys of the same mouse (**C**). All recipients were BRG. Donor strains are shown on the *x* axis. Recipient mono-DCs in grafts were measured as in Fig.1. (**A**) Mouse chromosome 2 region that differs between R7 and R12 congenic strains and contains the *Sirpa* locus. β_2m, β_2-microglobulin. (B) Comparison of innate alloresponses elicited by NOR or NOD grafts to those elicited by congenic donors that carry the NOR (R7) or NOD (R12) *Sirpa* allele. (C) Innate alloresponses elicited by grafts from NOD and R12 congenic mice transplanted into the same BRG recipient. (D) Grafts from BRG mice congenic for the NOD *Sirpa* allele (BRGS) induce the same alloresponse as those from NRG mice in BRG recipients. $n = 6$ to 7 mice per group per experiment. Except for the BRGS group, experiments were performed twice. Each dot represents an individual biological replicate. Bars are means. $^{**}P < 0.01$, $^{****}P < 0.0001$ (unpaired two-tailed *t* test).

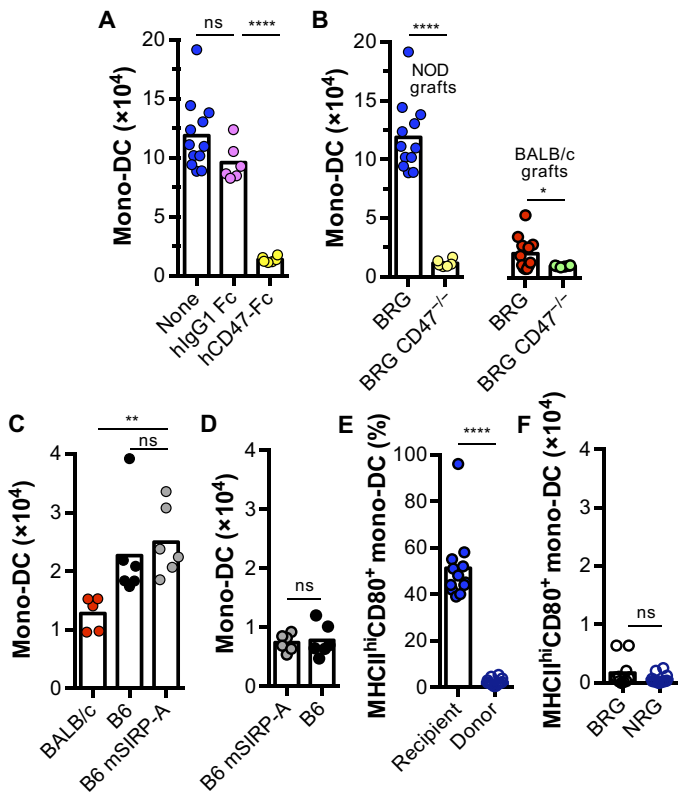

Fig. 4. Donor SIRPα binding to recipient CD47 is required for triggering the innate alloresponse. (**A**) NOD grafts were transplanted to BRG mice that received either hCD47-Fc, a decoy protein that binds to the NOD SIRPα variant and prevents it from binding to mouse CD47, or isotype control Fc protein (hIgG1 Fc). Recipient mono-DCs in grafts were measured as in Fig.1. Control untreated recipients (None) from previous experiments are shown for comparison. (**B**) NOD or BALB/c grafts were transplanted to BRG *CD47*$^{-/-}$ mice, and the innate alloresponse was compared with that of CD47-sufficient (BRG) recipients from previous experiments. (**C** and **D**) Grafts from wild-type (B6 and BALB/c) or mutant (B6 mSIRP-A) mice, which lack the intracellular signaling domain of SIRPα, were transplanted to CRG (C) or BRG (D) recipients to test the effect of removing SIRPα signaling from donor cells on the host response. (**E**) Proportion of mature (MHCII^{hi}CD80^{+}) recipient or donor mono-DCs in NRG allografts transplanted to BRG mice. (**F**) Absolute number of mature host mono-DC in allogeneic (NRG) versus syngeneic (BRG) grafts transplanted to BRG recipients. $n = 6$ mice per group per experiment. Experiments were performed once or twice. Each dot represents an individual biological replicate. Bars are means. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ (unpaired two-tailed *t* test).

the intracellular signaling function of SIRPα in donor cells did not influence the magnitude of the host innate alloresponse. We also ruled out the possibility that increased host mono-DC accumulation was an indirect consequence of donor monocyte activation by host cells (that is, a graft-versus-host reaction). As a measure of monocyte activation, we analyzed the maturation state of donor and host mono-DCs in NRG grafts transplanted to BRG mice. We found that only 2% of donor mono-DC had acquired a mature (MHCII^{hi}CD80^{+}) phenotype compared with 52% of recipient mono-DCs (Fig. 4E). Moreover, the number of mature donor mono-DCs in NRG grafts did not exceed that in syngeneic BRG grafts (Fig. 4F). Therefore, the host innate alloresponse to bone marrow grafts is a consequence of donor SIRPα binding to recipient CD47 and not the indirect result of a graft-versus-host reaction.

Mouse SIRPα amino acid polymorphism modulates binding to CD47

The results presented so far support a model in which polymorphism in the extracellular domains of donor SIRPα is recognized by host CD47 to identify cells as self or nonself. To test this model further, we investigated the extent and location of amino acid polymorphisms in mouse SIRPα by aligning the predicted SIRPα protein sequences from 19 different mouse strains whose genomes had been sequenced (www.sanger.ac.uk/science/data/mouse-genomes-project). We found that amino acid variability is largely restricted to the extracellular region and is most frequent in the N-terminal, CD47-binding IgV domain (Fig. 5A; alignment is shown in fig. S2) (*25*–*27*). Phylogenetic analysis revealed four unique IgV domains among the 13 common inbred mouse strains examined, whereas each of the six wild-derived

mouse strains had its own unique IgV sequence (Fig. 5B). The latter suggests the presence of a considerable number of mouse SIRPα alleles in the wild. We also observed that NOD and CAST strains, which elicited the strongest innate alloresponses in BRG mice (Fig. 1A), have closely related IgV domains, whereas NOR, which elicited a very weak alloresponse, shared the same IgV domain with B6 recipients (Fig. 5B and fig. S2). Because amino acid polymorphism in SIRPα's IgV domain influences binding to CD47 (*12*), we asked whether NOD and CAST SIRPα variants share greater binding to CD47 than SIRPα variants of mouse strains that induce weaker alloresponses. To answer this question, we compared the binding of mouse CD47-Fc (mCD47-Fc) fusion protein to splenic monocytes from NOD and CAST mice to that from B6, BALB/c, and C3H mice. Splenic monocytes constitutively express high levels of SIRPα (*16*). As shown in Fig. 5C, mCD47-Fc displayed greater binding to NOD and CAST cells than B6, BALB/c, and C3H cells at each concentration tested. SIRPα expression was comparable on monocytes from all strains tested (Fig. 5D), indicating that dissimilarities in binding were not due to differences in SIRPα cell surface density. The data therefore suggest that donor SIRPα IgV domain polymorphism controls the host innate alloresponse by modulating the binding of SIRPα to CD47.

Donor SIRPα polymorphism modulates monocyte proliferation

To further investigate the role of SIRPα polymorphism in monocyte activation, we immunized BRG mice with splenocytes from donors expressing SIRPα variants with different CD47-binding affinities and analyzed the proliferation of myeloid cells in the spleen 7 days later. Mice were injected with 5-ethynyl-2′-deoxyuridine (EdU) 1 hour before spleen harvest to label dividing cells in situ. As shown in Fig. 6A, significant EdU uptake was observed only in Ly6C^{hi} monocytes (and to a much lesser extent, in mono-DCs) in mice immunized with allogeneic, but not syngeneic, splenocytes. The magnitude of monocyte proliferation correlated with strength of SIRPα binding to CD47. Splenocytes from NOD mice, which express a high-affinity variant of SIRPα, induced the greatest proliferation; NOR splenocytes, which share the same lower-affinity SIRPα allele with B6, induced the least; and BALB/c splenocytes gave an intermediate result (Fig. 6B). Monocyte proliferation was makedly reduced in CD47-deficient recipients stimulated with NOD cells (Fig. 6B). Together, these data provide additional evidence that differential binding of donor SIRPα to CD47 modulates monocyte activation.

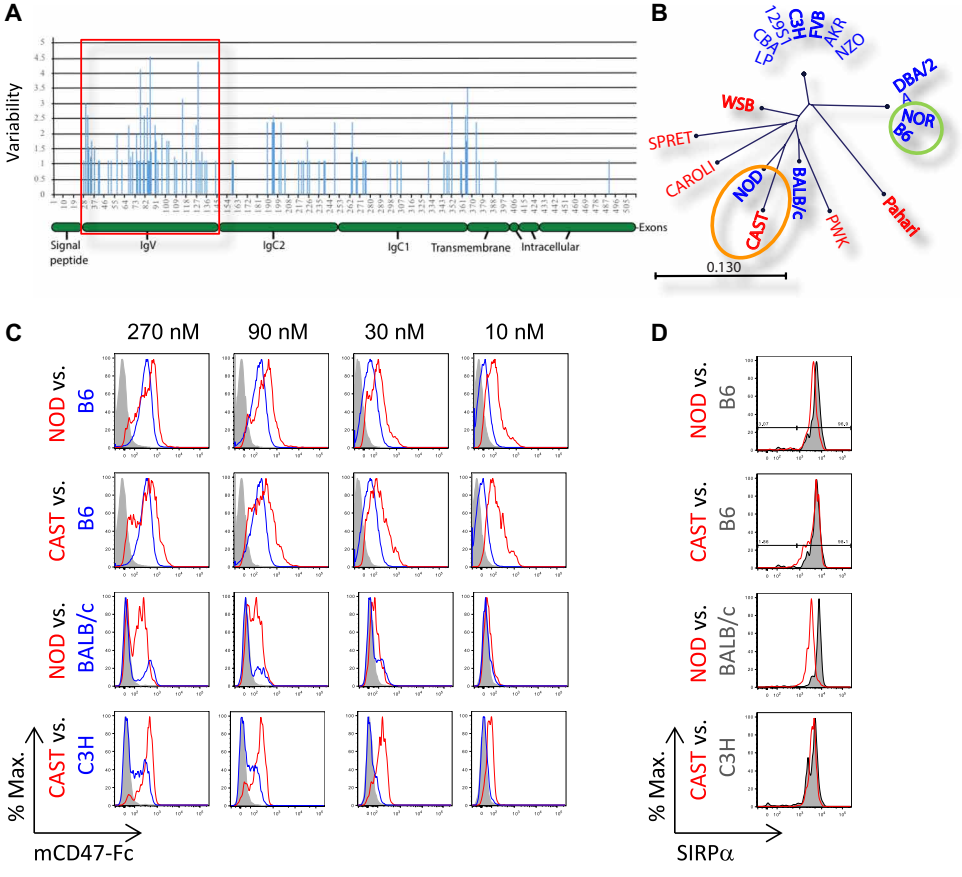

Fig. 5. Mouse SIRPα amino acid polymorphism modulates binding to CD47. (**A**) Amino acid variability in mouse SIRPα protein based on alignment of sequences from 19 mouse strains. Variability was calculated for each position along the sequence and aligned against the denoted SIRPα protein domains. IgV domain (red box) had the highest frequency of amino acid polymorphisms (number of vertical blue lines) and contained polymorphisms with the greatest degree of variability (height of vertical blue lines). (**B**) Phylogram representation of SIRPα IgV domain amino acid variation among the 19 mouse strains. Blue, common inbred strains; red, wild-derived inbred strains; bold, mouse strains tested in Fig.1; scale, proportion of amino acid substitutions; circles, mouse strains that share similar or identical CD47 IgV domains. (**C**) Binding of mCD47-Fc to splenic monocytes (Lin$^-$CD11b$^+$CD11c$^-$F4/80$^-$ cells) from NOD and CAST compared with B6, BALB/c, and C3H mice. Top: Serial dilution of mCD47-Fc. (**D**) SIRPα expression on monocytes from all strains tested. Histograms are representative of two to three biological replicates from two independent experiments (unpaired two-tailed *t* test).

Innate alloactivation is triggered by mismatch between donor and recipient SIRPα

Because SIRPα and CD47 are coexpressed on both donor and host cells, we tested the hypothesis that transplantation of allogeneic grafts carrying nonself *SIRPa* allele(s) causes innate immune activation by disturbing the balance between activating and inhibitory signals mediated by CD47 and SIRPα in host monocytes, respectively. This hypothesis would predict that under steady-state conditions or upon transplantation of a syngeneic graft, bidirectional interactions between CD47 and self-SIRPα are of equal affinity and therefore maintain monocytes in a quiescent state (Fig. 7A, top). The data shown in Fig. 7B are consistent with the prediction as they demonstrate that syngeneic grafts lacking CD47 elicit robust mono-DC infiltration, comparable with that triggered by allogeneic grafts. In the case of allogeneic transplantation, on the other hand, the introduction of donor cells, which express a nonself SIRPα variant

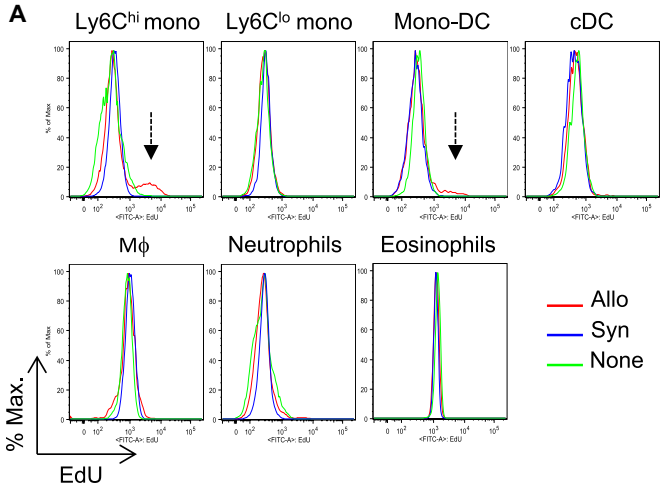

Fig. 6. Donor SIRPα polymorphism modulates monocyte proliferation. (**A**) BRG mice were immunized intraperitoneally with irradiated allogeneic (BALB/c) or syngeneic (B6) splenocytes. Spleen cells were analyzed 1 week later. Mice were pulsed with EdU 1 hour before spleen harvest. Representative flow plots of EdU staining of myeloid cell populations are shown. Arrows indicate EdU^+ cell population. cDC, conventional DC. (**B**) BRG or BRG *CD47*$^{-/-}$ mice were stimulated as in (A) with splenocytes from mouse strains shown on the *x* axis, and the proportion of EdU^+ cells in $Ly6C^{hi}$ monocyte subset was determined and divided by the proportion of EdU^+ cells in mice immunized with syngeneic (B6) splenocytes to determine the proliferation index. $n = 3$ mice per group per experiment × 2 experiments except for B6 group where total $n = 9$. Each dot represents an individual biological replicate. Bars are means. $^*P < 0.05$, $^{***}P < 0.001$ (unpaired two-tailed *t* test).

with greater binding to CD47 than recipient SIRPα, would be expected to upset the balance and cause monocyte activation (Fig. 7A, bottom). This is most evident in the case of NOD- and CAST-derived grafts, which express SIRPα variants with greater binding to CD47 than B6 SIRPα (Fig. 5C) and elicit robust innate alloresponses in B6 (BRG) mice (Fig. 1A). The hypothesis would also predict that performing allogeneic transplantation in the opposite direction, such that donor SIRPα has weaker binding to CD47 than recipient SIRPα, should suppress the innate alloresponse. This prediction is borne out by the results shown in Fig. 7C: Mono-DC accumulation in BRG grafts transplanted to NRG recipients was significantly less than that in NRG grafts transplanted to BRG recipients. Therefore, the experimental findings presented in this manuscript support a model in which the innate immune system senses allogeneic nonself by integrating activating and inhibitory signals delivered by CD47 and SIRPα, respectively.

DISCUSSION

We have identified a mechanism by which the innate immune system distinguishes between self and allogeneic nonself that is distinct from allorecognition by T or NK cells. Unlike allorecognition by T cells, this innate mechanism is not linked to the *Mhc* and is not based on polymorphisms in both donor and recipient molecules. Instead, it is mediated by differential binding of a polymorphic ligand in the donor, SIRPα, to a monomorphic receptor in the recipient, CD47. The SIRPα-CD47 pathway resembles allorecognition by NK cells in that it relies on the integration of stimulatory and inhibitory signals by the responding cell (*28*) but differs from it in several aspects. First, monocytes use the same molecular pair (CD47 and SIRPα) that engages each other in opposite directions to deliver stimulatory and inhibitory signals, whereas NK cells rely on distinct sets of stimulatory and inhibitory ligand-receptor pairs. Second, monocytes sense allogeneic nonself through subtle variations in the binding of SIRPα to CD47, whereas NK cells sense missing self-MHC molecules. Third, unlike NK cell allorecognition, the monocyte allorecognition mechanism elucidated here is not dependent on MHC mismatch between the donor and recipient but instead on mismatch in a gene not linked to the MHC. Last, allorecognition systems that predate the evolution of NK cells, T cells, and the MHC have been described in marine invertebrates (*29*). They also use polymorphic transmembrane proteins that contain Ig-like domains but have minimal homology to nonself recognition systems in vertebrates (*30*–*32*).

Inhibitory functions of SIRPα on myeloid cells have been well characterized (*16*, *33*). SIRPα engagement by CD47 suppresses macrophage phagocytic function (*18*, *34*) and DC maturation (*20*). CD47, in contrast, has stimulatory functions in immune cells. It provides costimulatory signals to T cells (*21*, *35*) and enhances DC homeostasis and migration (*36*). Consistent with these functions, CD47$^{-/-}$ mice were refractory to the induction of autoimmune diseases; for example, experimental allergic encephalomyelitis (*37*), experimental colitis (*38*), and murine lupus (*39*). In addition, Wong *et al.* showed that enhanced binding of SIRPα to CD47 in the NOD mouse is a key determinant of the pathogenesis of autoimmune diabetes, likely due to the costimulatory actions of CD47 on T cells (*12*). Together, these data support our proposal that engagement of CD47 on recipient monocytes by SIRPα on graft cells provides an activation signal that causes monocyte proliferation and differentiation. However, a caveat to our study is that it lacks direct proof that SIRPα polymorphism causes differential CD47 signaling in allostimulated monocytes. The intracellular signaling pathways triggered by CD47 in monocytes are not known and seem complex. On the basis of the studies in nonmyeloid cells, CD47 likely signals via G_i protein–dependent or G_i protein–independent pathways by associating with integrins in the cell membrane (*40*).

A role for CD47-SIRPα interactions in transplantation has been previously reported but only in the context of the long-held view that CD47 is a marker of self that, if altered or absent, triggers immune activation (*16*). Because CD47 is species-specific (*41*), xenografts activate host phagocytes because xenogeneic CD47 on the graft does not bind to host SIRPα (*42*). However, the phagocytic response is significantly diminished if the graft is made to express host CD47 (*43*) or if the host carries a SIRPα variant that binds xenogeneic CD47 with high affinity. The latter is exemplified by the ease of acceptance of human stem cells by immunodeficient NOD mice because of the exceptional binding of NOD SIRPα to human CD47 (*14*, *15*). CD47 expression on donor cells also plays a role in the alloimmune response. Wang *et al.* showed that the infusion of allogeneic spleen cells in mice results in the acceptance of a subsequent graft from the

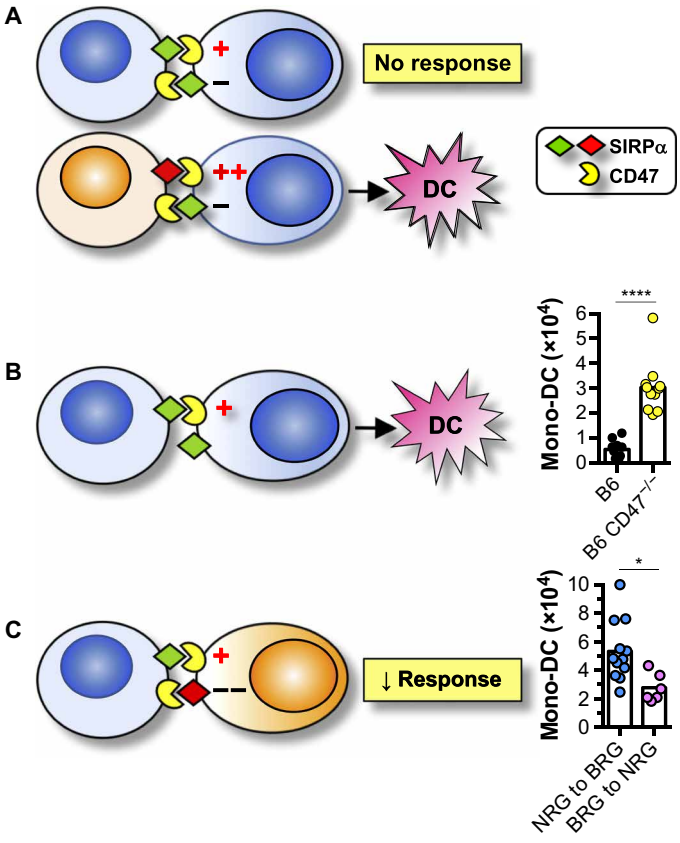

Fig. 7. Innate alloactivation is triggered by mismatch between donor and recipient SIRPα. (**A**) Top: Balance between activating signals (+) mediated by CD47 and inhibitory signals (–) mediated by SIRPα in recipient monocytes in the syngeneic transplantation setting. Bottom: Imbalance in the allogeneic setting when donor SIRPα (red) has greater affinity to CD47 than recipient SIRPα (green). The net result of this imbalance is recipient monocyte differentiation to mono-DC. (**B**) Imbalance is created if the syngeneic graft lacks CD47. Bar graph shows results from B6 CD47$^{-/-}$ and B6 wild-type grafts transplanted to separate BRG recipients. Graft-infiltrating mono-DCs were quantified as in Fig. 1. (**C**) Reversing direction of allotransplantation, such that donor SIRPα has weaker binding to CD47 than recipient SIRPα, inhibits the innate alloresponse. Experimental data are shown in bar graph. $n = 5$ to 6 mice per group per experiment. Experiments were performed once or twice. *$P < 0.05$, ****$P < 0.0001$ (unpaired two-tailed t test).

same donor but fails to do so if the spleen cells lacked CD47 due to activation of host DCs (*44*, *45*). In contrast, our work has identified an alternate function of the CD47-SIRPα pathway whereby the polymorphic partner, SIRPα, serves as a marker of allogeneic nonself on donor tissues that is detected by CD47 on host monocytes.

Although we did not establish in this study a formal association between donor SIRPα polymorphism or SIRPα-CD47 interactions and allograft outcomes, several lines of evidence suggest that this pathway is important in both bone marrow and solid organ transplantation. For example, CD47$^{-/-}$ recipients were found to accept xenogeneic and allogeneic hematopoietic stem cells more readily than wild-type mice and to develop less graft-versus-host disease (*23*, *46*). Conversely, CD47$^{-/-}$ donor cells failed to engraft because of the increased phagocytosis by recipient macrophages (*47*). Similar to bone marrow grafts, heart allografts from NOD donors harbored significantly more mature mono-DCs than allografts from NOR mice (*5*). These DCs were particularly adept at driving type 1 T cell responses that drive allograft rejection (*5*, *7*). Whether SIRPα polymorphism, which is also prevalent in the human population (*14*), modulates outcomes after bone marrow or solid organ transplantation remains to be seen. Another intriguing possibility is that SIRPα polymorphism may affect the maternal response to the allogeneic fetus or influence the pathogenesis of immune-mediated diseases of pregnancy. Therefore, exploring the role of SIRPα in natural (pregnancy) or artificial (transplantation) allogeneic encounters should yield important biological and clinical insights.

MATERIALS AND METHODS

Study design

We used a bone marrow plug transplantation model to conduct a genetic mapping study. Six biological replicates (six individual transplant recipients) per group were included in each experiment. Both sexes of mice were used. Experiments were repeated once in most instances, resulting in a total of 12 biological replicates. Sample sizes were not based on power analysis but on our previous experience that three to six biological replicates are sufficient to discern statistically significant differences between groups using the same readout (number of infiltrating host mono-DC) (*5*). Sample size was not altered at any time during the course of the study. A biological replicate was excluded only if the mouse died before the bone marrow plug could be harvested on day 7 after transplantation. This exclusion criterion was established prospectively, it occurred very rarely, and we did not experience any technical problems in harvesting or analyzing the grafts that would have led to exclusion of a biological replicate. All data points were included, and no outliers were excluded. All end points were prospectively selected. It was not possible to blind the study because of the need to identify donors and recipients, and because in many instances, the donor or recipient groups had different coat colors. The first investigator (H.D. or K.I.A.-D.) to analyze the flow cytometry data was not blinded, but randomly chosen subset of groups were reanalyzed blindly by either M.H.O. or F.G.L.

Mice

C57BL/6J (B6), B6.SJL-Ptprca Pepcb/BoyJ (B6.CD45.1), BALB/cJ (BALB/c), BALB.B, B6.C-H2^{d}/bByJ (B6.C), NOD/ShiLtJ (NOD), NOR/LtJ (NOR), DBA/2J (DBA), FVB/NJ (FVB), C3H/HeJ (C3H), *Mus pahari*/EiJ (Pahari), WSB-EiJ (WSB), CAST-EiJ (CAST), NOD.Cg-*Rag1*^{tm1Mom} *Il2rg*^{tm1Wjl}/SzJ (NRG), C;129S4-Rag2tm1.1Flv Il2rgtm1.1Flv/J (CRG), NOD.Cg-Prkdc^{scid} B2mtm1Unc/J (NOD.*Prkdc*^{scid}.*b2m*$^{-/-}$), B6.129S-Rag2tm1Fwa Cd47tm1Fpl Il2rgtm1Wjl/J [B6.*Rag2*$^{-/-}$*γc*$^{-/-}$ *Cd47*$^{-/-}$ (BRG *CD47*$^{-/-}$)], and B6.129S2-H2$^{dlAb1\text{-}Ea}$/J (B6.*Mhc2*$^{-/-}$) mice were purchased from the Jackson Laboratory (JAX). B6-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Wjl} (BRG) mice were purchased from Taconic. B6.129P-*Cx3cr1*^{tm1Litt}/J (B6 CX_3CR1-eGFP CD45.2) (JAX) mice were bred on the Rag2$^{-/-}$γc$^{-/-}$ background. C57BL/6.NOD-(*D2Mit447-D2Mit338*) Rag2$^{-/-}$γc$^{-/-}$ (BRGS) congenic mice were a gift from K. Takenaka and K. Akashi (Kyushu University, Fukuoka, Japan) (*15*). B6.*Sirpa*^{tm1} mice, which lack the SIRPα intracytosolic domain, were provided by J.S.I. and T.M. (*24*). All mice were maintained at the University of Pittsburgh Animal Facility under specific pathogen–free conditions. All animal procedures were performed with the approval of the Institutional Animal Care and Use Committee at the University of Pittsburgh.

Surgical procedures

Bone marrow plug transplantation was performed under the kidney capsule after isolating intact bone marrow plugs from donor femurs (*48*). Recipient mice were anesthetized, and the kidney was exposed via a small flank incision. A small incision was made in the kidney capsule, a pocket was created with blunt forceps, and a 4-mm bone marrow plug fragment was placed in the subcapsular pocket with vascular forceps.

Mouse treatment

To block NOD SIRPα-CD47 interaction, mice received 250 μg of recombinant hCD47-Fc chimera (hCD47-Fc) (R&D Systems) intraperitoneally for four times every other day starting on the day of transplantation. Control mice received an equivalent dose of recombinant hIgG1 Fc (R&D Systems). Both reagents were provided in endotoxin-free phosphate-buffered saline.

Mono-DC analysis by flow cytometry

Bone marrow plug grafts were removed from the kidney capsule, homogenized using a gentleMACS tissue processor (Miltenyi), and digested at 37°C for 45 min in RPMI plus 10% fetal calf serum containing collagenase IV (350 U/ml; Sigma-Aldrich) and deoxyribonuclease I (20 ng/ml; Sigma-Aldrich). Leukocytes were isolated by gradient centrifugation using Lympholyte-M (Cedarlane Laboratories). Total recovered cells were counted using a hemocytometer before staining with antibodies. Fluorochrome- or biotin-tagged antibodies were purchased from BD Pharmingen, eBioscience, BioLegend, or R&D Systems: CD90.2 (30-H12), CD45.1 (A20), CD45.2 (104), CD45R/B220 (RA3-6B2), CD49b (DX5), NK1.1 (PK136), F4/80 (BM8), CD11b (M1/70), CD11c (N418), Ly6G (1A8), CD19 (1D3), MHC II (M5/114.15.2), CD80 (16-10A1), H-2K^{d} (SF1-1.1.1), and H2-k^{k} (AF3-12.1.3). Fixable Viability Dye eFluor 506 was purchased from Affymetrix/eBioscience. EdU uptake was analyzed using the Click-iT assay according to the manufacturer's instructions (Thermo Fisher Scientific). Flow acquisition was performed on an LSRFortessa flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star Corp.). Recipient and donor cells were distinguished using congenic markers (CD45.1/2), green fluorescent protein expression, or H-2 expression (in the case of BALB/c grafts).

CD47-SIRPα binding assay

Flow-based mCD47-Fc (R&D Systems) binding assay was performed as previously described with some modifications (*12*). Briefly, spleen cells from indicated mouse strains were blocked with anti-CD16/32 (BD Biosciences), followed by preclustering of SIRPα with unconjugated P84 antibody (eBioscience). Cells were then incubated with serial dilutions of mCD47-Fc for 30 min, followed by secondary staining with anti–hIgG1–phycoerythrin (PE) (R&D Systems). Control cells were prepared in the same fashion except that hIgG1 Fc (R&D Systems) was used instead of mCD47-Fc. Flow cytometry was performed to measure mCD47-Fc binding to monocyte subset (CD45^{+}Lin^{-}Ly6G^{-}CD11b^{+}CD11c^{-}F480^{-} cells). SIRPα expression was performed by staining cells with PE-conjugated P84 antibody (BD Biosciences) under identical conditions as binding assay.

Mouse SIRPα sequence alignment

Whole mouse genomes were acquired from the Sanger Mouse Genome Project (www.sanger.ac.uk/science/data/mouse-genomes-project). A BLAST search was performed against the B6 *Sirp*a gene sequence to extract strain-specific *Sirpa* gene sequences from genomes of other strains. Gene sequences were translated to amino acid sequences and aligned to the B6 SIRPα sequence using CLC Genomics Workbench. Variability of amino acids along the SIRPα sequence was calculated as the number of different residues/frequency of the most common residue − 1. This measure of variability was plotted against the length of the SIRPα amino acid sequence to identify the most variable regions. A phylogram of SIRPα IgV domain amino acid sequences was compiled in CLC Genomics Workbench to represent which strains were most similar or dissimilar from each other.

Statistical analysis

All data points are shown in the graphs as scatterplots. Bars depict the means. GraphPad Prism 6 was used for statistical analyses. Statistical significance ($P < 0.05$) was calculated using the unpaired two-sided *t* test, and *P* values are shown in the graphs. All comparisons found to be significant by *t* test were also significant by the nonparametric Mann-Whitney test.

SUPPLEMENTARY MATERIALS

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Fig. S1. Gating strategy for identifying recipient-derived graft-infiltrating mono-DCs.
Fig. S2. Alignment of predicted SIRPα protein sequences from 19 mouse strains.
Table S1. Raw data file.

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Donor SIRPα polymorphism modulates the innate immune response to allogeneic grafts

Hehua Dai, Andrew J. Friday, Khodor I. Abou-Daya, Amanda L. Williams, Steven Mortin-Toth, Matthew L. Nicotra, David M. Rothstein, Warren D. Shlomchik, Takashi Matozaki, Jeffrey S. Isenberg, Martin H. Oberbarnscheidt, Jayne S. Danska and Fadi G. Lakkis

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Looking beyond MHCs in transplant rejection

Mice engineered to lack T, B, and NK cells generate mature dendritic cells in response to allogeneic transplants. Precisely how these mice recognize allografts to be "nonself" has remained a mystery. Using an elegant positional cloning approach, Dai *et al.* have identified polymorphisms in the mouse gene encoding signal regulatory protein α (SIRP α) to be key in this innate self-nonself recognition. They show that SIRPα receptor CD47 binds SIRPα variants with distinct affinities and propose this affinity sensing to be the mechanism that triggers dendritic cell maturation, the first step in the initiation of the alloimmune response. Given that the SIRP α gene is also polymorphic in humans, it remains to be seen whether human SIRP α variations influence transplantation success.

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