# Role for CD47-SIRP$\alpha$ signaling in xenograft rejection by macrophages

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**We have previously proven that human macrophages can phagocytose porcine cells even in the absence of Ab or complement opsonization, indicating that macrophages present a pivotal immunological obstacle to xenotransplantation. A recent report indicates that the signal regulatory protein (SIRP)$\alpha$ is a critical immune inhibitory receptor on macrophages, and its interaction with CD47, a ligand for SIRP$\alpha$, prevents autologous phagocytosis. Considering the limited compatibility (73%) in amino acid sequences between pig and human CD47, we hypothesized that the interspecies incompatibility of CD47 may contribute to the rejection of xenogeneic cells by macrophages. In the present study, we have demonstrated that porcine CD47 does not induce SIRP$\alpha$ tyrosine phosphorylation in human macrophage-like cell line, and soluble human CD47-Fc fusion protein inhibits the phagocytic activity of human macrophages toward porcine cells. In addition, we have verified that manipulation of porcine cells for expression of human CD47 radically reduces the susceptibility of the cells to phagocytosis by human macrophages. These results indicate that the interspecies incompatibility of CD47 significantly contributes to the rejection of xenogeneic cells by macrophages. Genetic induction of human CD47 on porcine cells could provide inhibitory signaling to SIRP$\alpha$ on human macrophages, providing a novel approach to preventing macrophage-mediated xenograft rejection.**

xenotransplantation | phagocytosis | reticuloendothelial system

The ability to transplant pig organs into humans would resolve the current crisis in the supply of cadaveric human organs for the treatment of end-stage organ failure (1, 2). Humans lack a functional $\alpha$1,3-galactosyltransferase (GalT) gene, and therefore do not express Gal$\alpha$1,3Gal$\beta$1,4GlcNAc (Gal) carbohydrate residues, and produce abundant natural antibodies (Abs) to the Gal epitope (3). These anti-Gal Abs are a major barrier to xenotransplantation of pig organs into humans, because hyperacute rejection and acute humoral rejection are initiated by their binding to Gal determinants that are ubiquitously present on porcine cells. The genetically engineered GalT-knockout pigs that no longer express Gal epitopes appear promising in conferring protection against this type of xenograft rejection (4). Following such a significant step forward, the subsequent barriers of a vigorous cellular immune response to xenografts are currently a major concern. The survival of hearts from GalT-deficient pigs in baboons can be markedly prolonged under a chronic immunosuppressive regimen, but these are eventually rejected (5). A promising approach to conquering such inevitable rejection involves inducing tolerance of xenoreactive T cells in pig-to-human transplant by achieving mixed hematopoietic chimerism (6).

In addition to T cells, the mononuclear phagocyte system plays a critical role in the vigorous cellular immune response toward xenografts. Macrophages mediate rapid rejection of porcine hematopoietic cells (7, 8) and pancreatic islets (9), which express little or no Gal antigen (10). After infusion into baboons, these cells are avidly scavenged by primate macrophages and perhaps other reticuloendothelial (RE) system cells (including liver Kupffer cells and splenic red pulp macrophages) (11). Based on the fact that macrophages can be activated by prophagocytic signaling pathways through activating receptors, such as Fc$\gamma$ and complement receptors, complement- and Ab-mediated phagocytosis by macrophages could be a mechanism for targeting opsonized porcine cells. The use of genetically engineered pigs deficient for the GalT gene and/or transgenic for human complement regulatory proteins might overcome such obstacles (12, 13). However, we have recently reported that human RE macrophages can phagocytose porcine cells in the absence of Ab or complement opsonization, and that removing Gal epitopes from porcine cells failed to prevent this phagocytosis (14). This result suggests that regulation of macrophages in human recipients might be needed to achieve successful engraftment of porcine islet xenografts or marrow grafts used for tolerance induction even if GalT-deficient pigs were used. However, the long-term use of macrophage-depleting reagents is unlikely to be acceptable in the clinical setting, even if nontoxic drugs could be developed, because macrophages play a critical role in initiating immune responses toward pathogens. The elucidation of mechanisms for phagocytizing unopsonized porcine xenogeneic cells by human macrophages should provide further insights into the understanding of the robust xenoreactivity of macrophages and may lead to the development of approaches for attenuating macrophage-mediated xenograft rejection.

CD47, known as integrin-associated protein, is a ubiquitously expressed 50-kDa cell surface glycoprotein that serves as a ligand for signal regulatory protein (SIRP)$\alpha$ (also known as CD172a, SHPS-1), an immune inhibitory receptor on macrophages. CD47 and SIRP$\alpha$ constitute a cell–cell communication system (the CD47-SIRP$\alpha$ system) that plays important roles in a variety of cellular processes including cell migration, adhesion of B cells,

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Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; FCM, flow cytometric; LCL, lymphoblastoid cell line; PBL, peripheral blood lymphocyte; PBMC, peripheral blood mononuclear cell; RE, reticuloendothelial; SIRP$\alpha$, signal regulatory protein $\alpha$.

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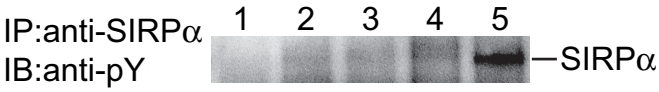


**Fig. 1.** SIRP$\alpha$ tyrosine phosphorylation in human macrophages was induced by incubation with human RBCs but not porcine RBCs. Differentiated THP-1 cells were incubated with human or porcine RBCs at 37°C in a water bath for 30 min. The cells were lysed, and the lysates were mixed with mouse anti-human SIRP$\alpha$ antibodies and 50% slurry of protein G-Sepharose beads by rotation at 4°C for 2 h. Precipitated proteins were separated by SDS/10% PAGE, followed by blotting to a nitrocellulose membrane. Rabbit immunoaffinity purified anti-phosphotyrosine IgG and goat anti-rabbit HRP-conjugated IgG were used as primary and secondary antibodies, respectively. Porcine RBCs alone (lane 1), human RBCs alone (lane 2), THP-1 cells incubated in medium alone (lane 3), or human macrophage cell line THP-1 cells incubated with porcine (lane 4) or human (lane 5) RBCs are shown. IP, immunoprecipitation; IB, immunoblotting; anti-pY, anti-phosphotyrosine.

and T cell activation (15–18). In addition, the CD47-SIRP$\alpha$ system is implicated in negative regulation of phagocytosis by macrophages. CD47 on the surface of several cell types (i.e., erythrocytes, platelets, or leukocytes) can protect against phagocytosis by macrophages by binding to the inhibitory macrophage receptor SIRP$\alpha$. The role of CD47-SIRP$\alpha$ interactions in the recognition of self and inhibition of phagocytosis has been illustrated by the observation that primary, wild-type mouse macrophages rapidly phagocytose unopsonized RBCs obtained from CD47-deficient mice but not those from wild-type mice (19). It has also been reported that through its SIRP$\alpha$ receptors, CD47 inhibits both Fc$\gamma$ and complement receptor-mediated phagocytosis (20). Considering the limited compatibility (73%) in amino acid sequences between pig and human CD47 (21), we hypothesized that interspecies CD47 incompatibility might contribute to the rejection of xenogeneic cells by human macrophages.

## Results

**Porcine CD47 Does Not Induce SIRP$\alpha$ Tyrosine Phosphorylation in Human Macrophage-Like THP-1 Cells.** In the CD47-SIRP$\alpha$ system, the ligation of SIRP$\alpha$ on macrophages by CD47 on target cells inhibits phagocytosis by promoting cytoplasmic domain phosphorylation of tyrosine and recruitment of Src homology 2 domain-containing protein tyrosine phosphatase-1, which is the major regulator of phagocytic responses (22). To determine whether porcine CD47 can interact with human SIRP$\alpha$, we assessed SIRP$\alpha$ tyrosine phosphorylation in a human macrophage cell line after contact with either porcine or human porcine RBCs. Western blotting revealed that the incubation of human macrophages with human RBCs resulted in detectable SIRP$\alpha$ tyrosine phosphorylation as expected (Fig. 1). Following incubation with porcine RBCs, SIRP$\alpha$ tyrosine phosphorylation was not induced in the human macrophage-like cell line above the level in control macrophages that were incubated with medium alone, indicating that porcine CD47 fails to induce SIRP$\alpha$ tyrosine phosphorylation in the human macrophage-like cell line. We also tested SIRP$\alpha$ tyrosine phosphorylation in the freshly isolated RE macrophages, because we considered that these were more physiologically and clinically relevant phagocytes. However, SIRP$\alpha$ tyrosine phosphorylation was barely detectable even after contact with human RBCs (data not shown).

**Soluble Human CD47-Fc Fusion Protein Partially Inhibits the Phagocytic Activity of Human Macrophages Toward Porcine Cells.** We next examined whether recombinant soluble human CD47-Fc fusion protein (which contains the extracellular domain of human CD47 fused to the Fc portion of human Ig), which itself drives an inhibitory signaling pathway to human macrophages through CD47-SIRP$\alpha$ interaction (23), could prevent the porcine cells from being phagocytosed by human macrophages. Because THP-1 cells did not vigorously phagocytose porcine cells in our preliminary study, human RE macrophages were used as effectors. Carboxyfluorescein succinimidyl ester (CFSE)-labeled porcine lymphoblastoid cell line (LCL) cells were used as targets in the phagocytosis assay, in which the macrophages engulfing target porcine cells could be identified by confocal microscopy and flow cytometric (FCM) analyses. Because of the limited availability of appropriate human samples, the phagocytic properties of human RE macrophages have not been extensively investigated. We isolated human RE macrophages from the perfusion effluents of liver allografts used in clinical liver transplantation. Freshly isolated human RE macrophages showed phagocytosis of porcine LCL cells in the absence of Ab and complement (Fig. 2), although unmanipulated peripheral monocyte/macrophages did not (data not shown). We have confirmed that porcine LCL cells express porcine CD47 by using mouse anti-human CD47 (BRIC126), which cross-reacts with porcine CD47, for FCM analysis [supporting information (SI) Fig. 5]. Because their expression levels on porcine LCL cells were somewhat lower than those on freshly isolated porcine peripheral blood lymphocytes or RBCs, we could not entirely exclude the possibility that the porcine LCL cells express CD47 at concentrations lower than those at which the "do not phagocytose" observation is made. However, in our previous study, porcine peripheral lymphocytes and RBCs expressing intact porcine CD47 were robustly phagocytosed by human RE macrophages, indicating that normal porcine CD47 signaling is ineffective (14). Mature resident macrophages differentiate from circulating monocytes and occupy the peripheral tissues and organs. These macrophages can employ a broad array of antimicrobial effector mechanisms, including phagocytosis of the pathogen. Hence, the difference in phagocytic activity against porcine cells between the peripheral monocyte/macrophages and RE macrophages is presumably derived from the differences in their maturation and anatomical environments. Nevertheless, in the presence of human CD47-Fc, the phagocytic activity of human RE macrophages toward porcine cells was partially but significantly suppressed (Fig. 2).

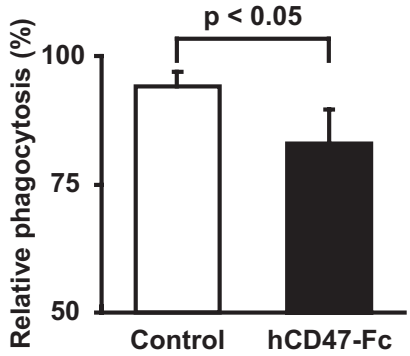


**Fig. 2.** Recombinant soluble human CD47-Fc partially protected the porcine cells from phagocytosis by human macrophages. Human RE macrophages were incubated with 10 $\mu$g of human IgG, Fc fragment (open bar), or 10 $\mu$g of recombinant soluble human CD47-Fc fusion protein (closed bar) for 5 min before the addition of CFSE-labeled porcine LCL cells. The macrophages that had phagocytosed the LCL cells were identified as CFSE and CD14 double-positive cells by FCM analysis. Data shown are normalized to the level of phagocytosis of LCL cells in the absence of Fc fragment. Data are the means ± SD for three separate experiments.

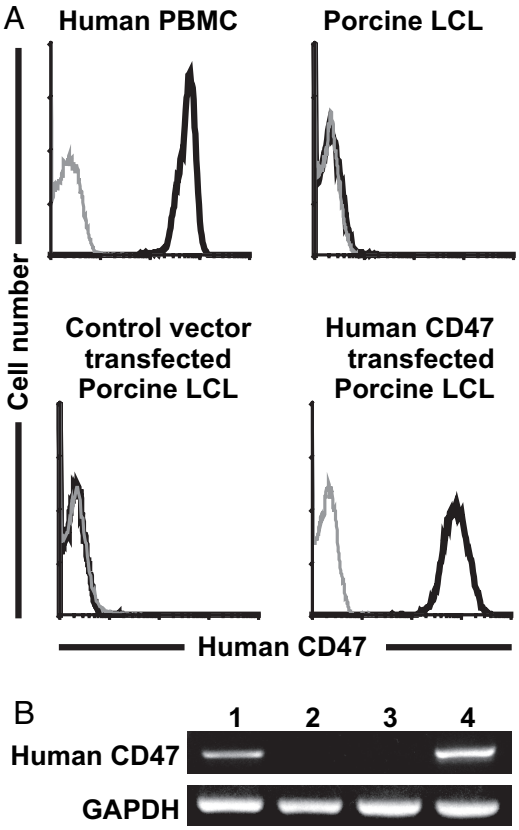


**Fig. 3.** Expression of human CD47 on a transfected porcine LCL was confirmed by FCM analysis and RT-PCR. (*A*) Representative histograms obtained by FCM analysis for human PBMCs, untreated porcine LCL cells, pKS336-transfected porcine LCL cells, and pKS336-human CD47-transfected porcine LCL cells are shown. Thin and bold lines represent staining with isotype control and anti-human CD47 mAb, respectively. (*B*) Expression of human CD47 on transfected porcine LCL was confirmed by RT-PCR using a specific pair of primers. Lane 1, human PBMC; lane 2, nontransfected porcine LCL; lane 3, pKS336-transfected porcine LCL; lane 4, pKS336-human CD47-transfected porcine LCL. GAPDH was used as a DNA loading control.

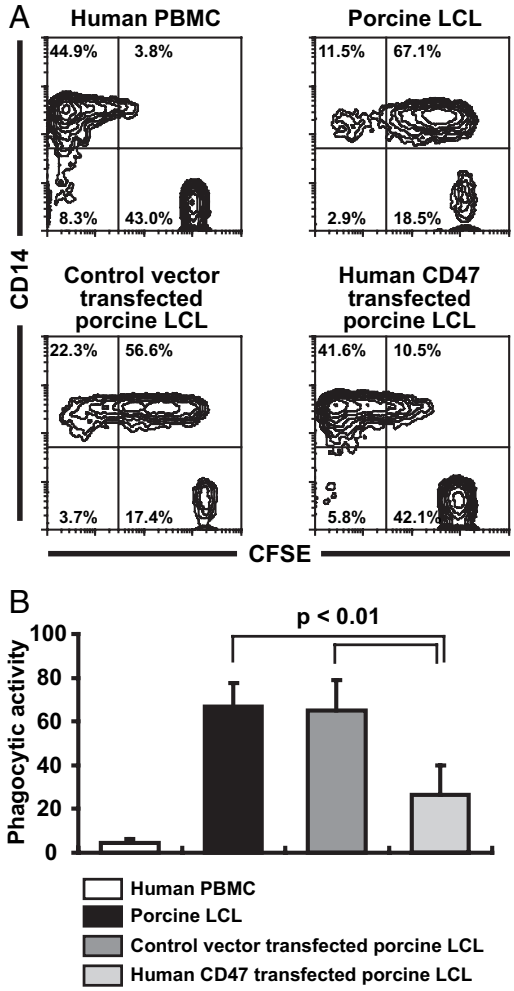


**Fig. 4.** Human CD47-expressing porcine LCL cells attenuate phagocytosis by human RE macrophages. (*A*) CFSE-labeled human PBMC, porcine LCL cells, pKS336-transfected porcine LCL cells, and pKS336-human CD47-transfected porcine LCL cells were incubated with RE macrophages for 4 h at 37°C. Macrophages counterstained with allophucocyanin-conjugated anti-human CD14 and phagocytosis of CFSE-labeled targets were measured by FCM analysis. Representative FCM profiles are shown. Regions of nonphagocytosing macrophages are shown in the upper left quadrants, regions of phagocytosing macrophages are shown in the upper right quadrants, and regions of residual targets are shown in the lower right quadrants. Percentages of total cells in each quadrant are shown. (*B*) Phagocytic activity was calculated by the following formula: phagocytic activity = (percentage in upper right quadrant/percentage in upper left quadrant + percentage in upper right quadrant) × 100. Data are the means ± SD for four separate experiments.

**Human CD47 Expression on Porcine Cells Radically Reduced the Susceptibility of These Cells to Phagocytosis by Human Macrophages.** To further determine whether human CD47 expression on porcine cells could efficiently prevent their phagocytosis by human RE macrophages, we generated human CD47-expressing porcine cell lines by transfecting porcine cells with a human CD47-expressing plasmid. The human CD47-expressing plasmid was prepared by inserting full-length human CD47 cDNA into the expression vector pKS336. LCL cells were transfected with either pKS336-human CD47 or the empty plasmid, and stable cell lines were obtained by blasticidin S selection. Human CD47 expression on the transfected porcine LCL cells was confirmed by FCM analysis and RT-PCR (Fig. 3). The pKS336-human CD47 vector-transfected porcine LCL cells expressed human CD47 on their surface, resembling human peripheral blood mononuclear cells (PBMCs), whereas both untreated porcine LCL cells and the control vector-transfected porcine LCL cells tested negative for human CD47. These LCL cells were compared for effects on the phagocytic activity of human RE macrophages. The macrophages that phagocytosed the target cells could be identified as CD14 and CFSE double positive cells (Fig. 4). As expected, human RE macrophages did not phagocytose human PBMCs, even in an allogeneic combination. In contrast, human RE macrophages actively phagocytosed porcine LCL cells without opsonization. However, human CD47 expression on porcine cells radically reduced the susceptibility of these cells to phagocytosis by human RE macrophages. Compared with the inhibiting effect of soluble human CD47-Fc on phagocytic activity of human RE macrophages toward porcine cells, that of human CD47 expression on target porcine cells was much more profound, probably reflecting more efficient CD47-SIRP$\alpha$ signaling via cell–cell contact. These results indicate that the interspecies CD47 incompatibility significantly contributes to the rejection of xenogeneic cells by macrophages and that the genetic manipulation of porcine cells for human CD47 expression could partially overcome such macrophage-mediated xenograft rejection.

## Discussion

It has been reported that CD47-SIRP$\alpha$ interactions exhibit limited cross-species reactivity probably because of species-

specific posttranslational modifications of CD47 such as glycosylation, i.e., CD47 on pig but not mouse, cow, or rat RBCs bind the recombinant extracellular dominant of human SIRP$\alpha$1 (24). In addition, we have recently demonstrated that pig CD47 does not interact with mouse SIRP$\alpha$ (25). However, it remained to be elucidated whether pig CD47-human SIRP$\alpha$ interactions could deliver a signal inhibiting engulfment or not. In the present study, we have proven that porcine CD47 fails to induce SIRP$\alpha$ tyrosine phosphorylation in human macrophage-like THP-1 cells and does not inhibit engulfment by human macrophages toward porcine cells.

Although macrophages can be activated by prophagocytic signaling pathways through activating receptors, such as Fc$\gamma$ and complement receptors, their phagocytic activity is also controlled by the signal strength of immune inhibitory receptors (20). Besides Fc$\gamma$ and complement receptors, it has been postulated that lectin-mediated carbohydrate binding provides activating signals to macrophages without opsonization (26). A recent study has shown that Galectin-3, an ≈30-kDa lectin composed of a terminal carbohydrate recognition domain, which is responsible for the specific recognition of $\beta$-galactose [Gal$\beta$(1-3/4) GlcNAc] and an N-terminal domain, is expressed in human macrophages (27). This lectin has been shown to be a receptor for xenoantigens, including not only $\alpha$-Gal but also *N*-glycolylneuraminic acid, which is expressed on porcine cells. Galectin-3 binds to porcine cells much more strongly than to human cells. GalT-deficient pigs may not eliminate macrophage-mediated rejection because removal of the GalT enzyme could leave many of the *N*-acetyllactosamine structures uncapped, as seen in GalT-deficient mice (28). It is not yet clear whether inhibitory signals such as CD47-SIRP$\alpha$ will override all activating signals delivered to macrophages by xenoantigens. In this study, the CD47-SIRP$\alpha$ inhibitory signal appeared to overcome whatever activating signal for macrophage phagocytosis was being delivered. Although inhibitory signaling seems to be predominant, elimination of xenoantigen-induced activating signals may also be required to completely abolish phagocytic activity of human macrophages toward porcine cells, consistent with our finding that human CD47 expression on porcine cells markedly reduced but did not completely eliminate the susceptibility of these cells to phagocytosis by human RE macrophages.

In the present study, soluble human CD47-Fc fusion protein partially inhibits the phagocytic activity of human RE macrophages toward porcine LCL cells. However, the reason for the solubility of CD47-Fc not being a comparably effective blocker against macrophage phagocytosis to the recombinant expression of human CD47 can only be speculated. Either the cross-linking required for CD47 signaling or CD47 have to be expressed on the same cells for the protective effect against phagocytosis. To address these possibilities, we have attempted mixing human RBCs expressing intact human CD47 with LCL cells to examine whether this will prevent phagocytosis. Even when human RE macrophages were constantly in contact with human RBCs, they continued to display unabated phagocytic activity against porcine LCL cells (SI Fig. 6), suggesting that CD47 expression on the target cells is critical for the complete protective effect. It might be possible that CD47-Fc nonspecifically binds to the cellular membrane of LCL cells and provides a signal that partially protects these cells against phagocytosis by RE macrophages.

It is well known that innate immune responses mediated by monocytes/macrophages may drive and shape the process of adaptive immunity. Phagocytic activities of macrophages are a first line of defense against invading infectious microbes, and the phagocytosed macrophages can present antigen derived from such foreign pathogens to T cells. It is likely that these mechanisms also take place in xenotransplantation from phylogenetically distant species. Therefore, specific elimination of phagocytic activity of human macrophages toward porcine cells by genetically inducing human CD47 might also attenuate subsequent T cell immune responses against porcine antigens while maintaining normal responses against other pathogens.

In conclusion, we have demonstrated a lack of cross-reactivity between a major macrophage inhibitory receptor between pig and human. This discovery provides further insight into on understanding of the robust xenoreactivity of macrophages, and may lead to the development of approaches for attenuating macrophage-mediated xenograft rejection, i.e., the genetic manipulation of porcine cells for human CD47 expression could provide a novel approach for preventing macrophage-mediated xenograft rejection.

## Materials and Methods

**Cell Cultures.** All cells were maintained at 37°C under a humidified atmosphere of 5% $CO_2$ in air. A porcine LCL was kindly provided by Christene Huang (Harvard Medical School). Cells were cultured in RPMI medium 1640 containing 10% FCS with 5 $\mu$M 2-mercaptoethanol (2-ME) (Katayama, Osaka, Japan), 10% Hepes buffer (Gibco, Grand Island, NY), and 100 units/ml penicillin-100 $\mu$g/ml streptomycin (Gibco). CHO-Ras-hCD47 cells producing human CD47-Fc were kindly provided by T. Matozaki (Gunma University, Gunma, Japan) (16). CHO-Ras-hCD47 cells were cultured in $\alpha$MEM (Sigma–Aldrich, St. Louis, MO) supplemented with 2 mM L-glutamine, 10 mM Hepes, 10% FCS, 100 units per ml penicillin/100 $\mu$g/ml streptomycin, 500 $\mu$g/ml Geneticin, and 500 $\mu$g/ml Zeocin. The human CD47-Fc fusion protein was then purified from such culture supernatants by column chromatography on recombinant Protein G (Amersham Pharmacia Biotech, Piscataway, NJ). Human IgG, Fc fragment (Chemicon, Temicula, CA) was used as control. The human macrophage cell line THP-1 (ATCC, Manassas, VA) was cultured in RPMI medium 1640 containing 10% FCS with 5 $\mu$M 2-ME, 10% Hepes buffer, and 100 units per ml penicillin/100 $\mu$g/ml streptomycin. Differentiation of THP-1 cells was achieved in 100 ng/ml phorbol myristate acetate for 2 days and confirmed by attachment of these cells to tissue-culture plastic.

**Expression Vectors and Transfection to LCL Cells.** The entire coding region of the CD47 cDNA was PCR-amplified from reverse-transcribed human lymphocyte cDNA with primers (sense) 5′-TGGACTCGACCATGTGGCCCCTGGTAGC-3′ and (antisense) 5′-GGAGCGGCCGCCTATTATTCATCATTCATC-3′. Amplified PCR product was digested with XhoI/NotI and cloned into pKS336 vector (kindly provided by M. Saijo, National Institute of Infectious Diseases, Tokyo, Japan) (29), which had been predigested with the same restriction endonucleases (SI Fig. 7), and the sequence was verified by using an ABI PRISM 3100 Sequencer (PE Applied Biosystems, Foster City, CA). LCL cells were transfected with pKS336-hCD47 or pKS336 (control transfection) by using DMRIE-C Reagent (Invitrogen, Carlsbad, CA) as described by the manufacturer. Stable transfectants were selected by culturing in RPMI containing blasticidin S (Invitrogen). Cell surface expression of CD47 was confirmed by immunofluorescence labeling and FACS analysis using anti-human CD47 mAbs [B6H12 (BD PharMingen, San Diego, CA) or BRIC126 (Serotec Ltd., Oxford, U.K.)].

**RT-PCR Analysis.** Total RNA was isolated from human PBMC, porcine LCL, and transfected porcine LCL, according to standard procedures by using ISOGEN (NIPPON GENE CO., LTD., Tokyo, Japan), and cDNA was synthesized with a First-Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ). The primers used for the detection of human CD47 were 5′-TATACTCCTGTTCTGGGGAC-3′ and 5′-TGGTATA-CACGCCGCAATAC-3′. As a control for the presence of amplifiable RNA, GAPDH primers were used (sense, 5′-TATACTAATGTTCTGGGGAC-3′; and antisense, 5′-TGG-

TATACACGCCGCAATAC-3′). Amplified PCR products were analyzed by agarose gel (10%) containing ethidium bromide.

**Isolation of Human RE Macrophages.** Human RE macrophages were isolated as described (14). In brief, the mononuclear cells were isolated from the perfusion effluents of liver allografts for clinical liver transplantation by gradient centrifugation with Separate-L (Muto Pure Chemicals Co., Ltd, Tokyo, Japan). Human RE macrophages were purified by using Monocyte Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) and a magnetic activated cell sorting system in accordance with the manufacturer's instructions. The purity of $CD14^+$ macrophages was confirmed by FCM analysis immunofluorescence by using FACSCalibur (Becton Dickinson, Mountain View, CA). More than 95% of the cells demonstrated positivity for the CD14 antigen. Without any preculture, freshly isolated human RE macrophages were immediately subjected to the phagocytosis assays. Ethical approval for this study was obtained from the Ethics Committee at the Hiroshima University Hospital. Informed consent was obtained from all donors for participation in this study.

**Immunoprecipitation and Immunoblotting.** Differentiated THP-1 cells ($2 \times 10^6$) were incubated in serum-free medium for 12 h before experiments and rinsed once with PBS. Then, $2 \times 10^7$ human or porcine RBCs, which were suspended in 2 ml of serum-free Iscove's modified DMEM (IMDM) supplemented with 2 mM sodium pervanadate (Sigma–Aldrich), were added into the macrophage cultures and incubated in a 37°C water bath for 30 min. The cells were lysed in 0.4 ml of lysis buffer [50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% protease inhibitor mixture (Sigma–Aldrich), and 2 mM sodium pervanadate]. For immunoprecipitation, the lysates were mixed with mouse anti-human SIRP$\alpha$ antibodies and a 50% slurry of protein G-Sepharose beads by rotation at 4°C for 2 h. Precipitated proteins were separated by SDS/10% PAGE, followed by blotting to a nitrocellulose membrane. Rabbit immunoaffinity-purified anti-phosphotyrosine IgG (Upstate, Charlottesville, VA) and goat anti-rabbit HRP-conjugated IgG (Santa Cruz Biotechnology, Santa Cruz, CA) were used as primary and secondary antibodies, respectively.

**Phagocytosis Assay.** Target cells were stained with the fluorescent dyes 5/6-CFSE (Molecular Probes, Eugene, OR) according to the manufacturer's protocol. After CFSE labeling, dead cells were removed from the target cells by using a Dead Cell Removal Kit (Miltenyi Biotec, Auburn, CA) and a magnetic activated cell sorting system in accordance with the manufacturer's instructions. CFSE-labeled target cells ($4 \times 10^5$) were incubated with human RE macrophages ($4 \times 10^5$) in 96-well polystyrene tissue culture plates (BD Labware, Franklin Lakes, NJ) in the absence of antibodies and complement factors. In some experiments, various volumes of autologous human RBCs were added. The macrophages engulfing target porcine cells could be identified as CFSE-labeling cells by FCM analyses. This was also confirmed by confocal microscopy (SI Fig. 8). The cells were harvested at the indicated times and stained with allophucocyanin-conjugated mouse anti-human CD14 (M5E2) (BD PharMingen) before FCM analysis. Assays were performed in triplicate and repeated on at least three different days using different macrophage donors.

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