CD47 cross-dressing by extracellular vesicles expressing CD47 inhibits phagocytosis without transmitting cell death signals

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Short title: CD47 crossdressing and function

Abstract

Transgenic CD47 overexpression is an encouraging approach to ameliorating xenograft rejection and alloresponses to pluripotent stem cells, and the efficacy correlates with the level of CD47 expression. However, CD47, upon ligation, also transmits signals leading to cell dysfunction or death, raising a concern that overexpressing CD47 could be harmful. Here, we unveiled an alternative source of cell surface CD47. We showed that extracellular vesicles (EVs), including exosomes (Exos), released from normal or tumor cells overexpressing CD47 (transgenic or native) can induce efficient CD47 cross-dressing on pig or human cells. Like the autogenous CD47, CD47 cross-dressed on cell surfaces is capable of interacting with SIRP α to inhibit phagocytosis. However, ligation of the autogenous, but not cross-dressed, CD47 induced cell death. Thus, CD47 cross-dressing provides an alternative source of cell surface CD47 that may elicit its anti-phagocytic function without transmitting harmful signals to the cells. CD47 cross-dressing also suggests a previously unidentified mechanism for tumor-induced immunosuppression. Our findings should help to further optimize the CD47 transgenic approach that may improve outcomes by minimizing the harmful effects of CD47 overexpression.

Keywords: CD47, extracellular vesicles, phagocytosis, apoptosis, cancer, xenotransplantation

Introduction

CD47 is ubiquitously expressed and acts as a ligand of signaling regulatory protein (SIRP) α , a critical inhibitory receptor on macrophages and dendritic cells (DCs). Emerging evidence indicates that the CD47-SIRP α signaling pathway plays an important role in regulation of macrophage and DC activation, offering a promising intervention target for immunological disorders. CD47KO cells are vigorously rejected by macrophages after infusion into syngeneic wild-type (WT) mice, demonstrating that CD47 provides a “don't eat me” signal to macrophages (Oldenborg et al., 2000, Wang et al., 2007a). Xenotransplantation using pigs as the transplant source has the potential to resolve the severe shortage of human organ donors, a major limiting factor in clinical transplantation (Yang and Sykes, 2007). We reported that the strong rejection of xenogeneic cells by macrophages (Abe et al., 2002) is largely caused by the lack of functional interaction between donor CD47 and recipient SIRP α (Wang et al., 2007b, Ide et al., 2007, Navarro-Alvarez and Yang, 2014). These findings led to the development of human CD47 transgenic pigs that have achieved encouraging results in pig-to-nonhuman primate xenotransplantation (Tena et al., 2017, Nomura et al., 2020, Watanabe et al., 2020). In addition to macrophages, a sub-population of DCs also expresses SIRP α (Wang et al., 2007a, Guillems et al., 2016). Importantly, CD47-SIRP α signaling also inhibits DC activation and their ability to prime T cells, and plays an important role in induction of T cell tolerance by donor-specific transfusion (DST) and hepatocyte transplantation (Wang et al., 2007a, Wang et al., 2014, Zhang et al., 2016). Thus, transgenic expression of human CD47 in pigs may also attenuate xenoimmune responses by ameliorating DC activation and antigen presentation. More recently, transgenic overexpression of CD47 was also applied for reducing allogenicity and generating hypoinmunogenic pluripotent stem cells (Han et al., 2019, Deuse et al., 2019).

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60 It has become increasingly evident that the CD47-SIRP α pathway plays a critical role in
61 containing anti-tumor immune responses. CD47 upregulation was detected in various cancer
62 cells, serving a powerful mechanism of evading macrophage killing (Jaiswal et al., 2009, Chan et
63 al., 2009). Accordingly, treatment with CD47 blockade could inhibit tumor growth via
64 macrophage-mediated mechanism (Jaiswal et al., 2009, Willingham et al., 2012, Weiskopf et al.,
65 2016, Chao et al., 2010, Liu et al., 2015a). More recently, the antitumor activity of CD47
66 blockade was found to be associated with CD11c⁺ DC activation and largely T cell-dependent
67 (Liu et al., 2015b, Chen et al., 2020, Li et al., 2020). Taken together, these studies revealed
68 clearly that the CD47-SIRP α pathway provides a powerful negative regulation for both innate
69 and adaptive immune responses and is increasingly considered as an effective intervention target
70 for protecting against transplant rejection and unleashing immune responses to cancer.

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72 Although negative regulation of immune responses is predominantly mediated by inhibitory
73 CD47-SIRP α signaling in macrophages and DCs, it remains largely unknown how transgenic
74 CD47 on pig or human pluripotent stem cells and upregulated CD47 on tumor cells interacts
75 with its receptor and ligands. In the present study, we identified an alternative source of cell
76 surface CD47. We found that extracellular vesicles (EVs), including exosomes (Exos) from cells
77 transgenically overexpressing CD47 or tumor cells overexpressing endogenous CD47, could
78 induce CD47 cross-dressing on pig or human cells. CD47 cross-dressed on cell surfaces can
79 interact with SIRP α to inhibit phagocytosis. However, unlike the autogenous CD47 that, upon
80 ligation, induces cell apoptosis and senescence (Mateo et al., 1999, Gao et al., 2016, Meijles et
81 al., 2017), ligation of CD47 cross-dressed on cell surfaces is not harmful to cells. This study

provides deeper insight into the effect of CD47 overexpression, which needs to be considered when designing strategies for gene-editing in pigs for xenotransplantation or in human pluripotent stem cells for cell replacement therapy, and for developing CD47 blockade-based cancer immunotherapy.

Results

Transgenic hCD47 cross-dressing in pig cells

CD47 cross-dressing was first identified by detecting of hCD47 on pig cells that were co-cultured with pig cells expressing transgenic hCD47. In these experiments, cell co-cultures were performed using cell line cells derived from porcine aortic cells (PAOC; **Figure S1**). First, we co-cultured parental PAOCs (expressing pig CD47; referred to as PAOC/CD47^p) with PAOCs that were genetically modified to express hCD47 isoform 2 (referred to as PAOC/CD47^{p/h2}) or isoform 4 (referred to as PAOC/CD47^{p/h4}). Flow cytometry analysis using anti-CD47 antibodies recognizing both human and pig CD47 revealed that PAOC/CD47^{p/h2} or PAOC/CD47^{p/h4} cells expressed a markedly increased level of CD47 compared to PAOC/CD47^p cells, and that PAOC/CD47^p cells showed significantly increased CD47 staining after co-culture with PAOC/CD47^{p/h2} or PAOC/CD47^{p/h4} (**Figure 1A**). These results suggest that PAOC/CD47^p cells were cross-dressed by CD47, likely transgenic hCD47, from PAOC/CD47^{p/h} cells during cultures. To confirm this possibility, we made CD47-deficient PAOC cells (via targeted deletion using CRISPR-Cas9- technology; referred to as PAOC/CD47^{null}) and PAOC47^{null} cells that express transgenic hCD47 (PAOC/CD47^{h2}). When the two PAOC cell line cells were co-cultured for 24 h, we found that PAOC47^{null} cells became positively stained by both anti-h/pCD47 (**Figure 1B**,

top) and anti-hCD47 (**Figure 1B, bottom**) antibodies. To rule out the possibility that, during the co-culture, the CD47null cells did not become CD47+, but PAOC/CD47^{h2} cells reduced hCD47 expression, we labeled PAOC/CD47^{null} (**Figure 1C, top**) or PAOC/CD47^{h2} (**Figure 1C, bottom**) cells with fluorescence Celltrace violet, and then cocultured the labeled cells with unlabeled PAOC/CD47^{h2} or PAOC/CD47^{null} cells, respectively. This experiment, in which fluorescence-labeling allowed for better distinguishing between the two cell populations in the cocultures, further confirmed that PAOC/CD47^{null} cells can be cross-dressed by CD47 after coculture with PAOC/CD47^h cells (**Figure 1C**).

Figure S1. Generation of CD47-deficient and hCD47-transgenic PAOC cell lines. (A) Schematic of pig CD47 loci and guide RNA sequence targeting the exon 2 of pig CD47 gene used for generating POAC/CD47^{null} cells. (B) Schematics of human CD47 isoform 2 (with 16aa intracellular domain (ID); top) and isoform 4 (with 34aa ID; bottom) plasmids, which were used for making POAC/CD47^{h2} and POAC/CD47^{h4} cells, respectively. (C) FACS profiles showing staining of the indicated PAOC cell lines with anti-h/p CD47 (top) and anti-hCD47 (bottom) antibodies.

Figure S2. SIRPα expression on pig cells. Shown are representative FACS profiles of PAOC47^{null} (Left) and pig LCL (Right) cells stained with anti-pig SIRPα.

PAOC cells express SIRPα (**Figure S2, left**) and pig SIRPα is reported to interact with human CD47 (Boettcher et al., 2019). Thus, to determine whether hCD47 cross-dressing is mediated by binding of hCD47 to pig SIRPα, we performed cocultures with pig LCL cells that do not express SIRPα (**Figure S2, right**). LCL cells cocultured with LCL cells that express hCD47 (LCL/CD47^{p/h}) (Ide et al., 2007, Wang et al., 2011), but not those cultured alone or mixed with LCL/CD47^{p/h} immediately prior to FACS analysis, were positively stained by anti-hCD47 antibodies (**Figure 1D**), indicating that hCD47 cross-dressing is SIRPα-independent.

We then wished to determine if cells other than the PAOC cell lines could be a source of CD47 for cross-dressing. Towards this end, PAOC47^{null} cells were cocultured with bone marrow cells from hCD47-transgenic miniature swine. Like pig cells cocultured with hCD47-transgenic cell lines (**Figure 1A-D**), PAOC47^{null} cells also became positively stained by anti-hCD47 antibodies after being cocultured with hCD47-tg swine cells (**Figure 1E**).

Fig 1. Transgenic hCD47 cross-dressing on pig cells. (A) PAOC/CD47^P cells and PAOC/CD47^{P/h2} or PAOC/CD47^{P/h4} cells were cultured alone or cocultured for 24h, and hCD47 cross-dressing on gated PAOC/CD47^P cells was assessed by FACS using anti-h/pCD47-PE mAb (clone CC2C6, reacting with both human and pig CD47). Shown are representative histogram profiles (Left; the numbers in the figure indicate the MFI of gated PAOC/CD47^P cells), and average MFI (Right; mean $\pm$ SDs; n = 6 replicates per group) of gated PAOC/CD47^P cells in the indicated cell cultures. ****, p < 0.0001 (two-tailed unpaired t-test). Results shown are representative of 3 independent experiments. (B) PAOC/CD47^{null} and PAOC/CD47^{h2} or PAOC/CD47^{h4} were cultured alone or cocultured for 24h, and analyzed for hCD47 cross-dressing on gated PAOC/CD47^{null} cells by FACS using anti-h/pCD47-PE mAb (top) or anti-hCD47-BV786 mAb (bottom). Shown are representative histogram profiles (Left panel; the numbers in the figure indicate the MFI of gated PAOC/CD47^{null} cells), and average MFI (Right panel; mean $\pm$ SDs; n = 3 replicates per group) of gated PAOC/CD47^{null} cells in the indicated cell cultures. **, p < 0.01; ****, p < 0.0001 (two-tailed unpaired t-test). Results shown are representative of 3 independent experiments. (C) Celltrace violet labeled PAOC/CD47^{null} (Left panel) or PAOC/CD47^{h2} (Right panel) were cultured alone (Violet CD47^{null} or Violet CD47^{h2}) or cocultured with unlabeled PAOC/CD47^{h2} (Violet CD47^{null} + CD47^{h2}) or PAOC/CD47^{null} (CD47^{null} or Violet CD47^{h2}) respectively, then the cells were stained by anti-h/pCD47-PE mAb. Shown are representative FACS profiles (n=3 replicates). Results shown are representative of 2 independent experiments. (D) Pig LCL and hCD47-tg LCL (LCL/CD47^{P/h}) cells were cultured alone or cocultured for 24h, and analyzed for hCD47 cross-dressing on gated LCL cells (the numbers in the figure indicate the MFI of gated LCL cells). The staining control of “mixed at staining” indicates the two types of cells were cultured separately and mixed immediately prior anti-CD47 staining. Two independent experiments were performed, and each experiment had 2 replicates per group. Representative FACS profiles are shown. (E) PAOC47^{null} cells were cocultured with bone marrow cells from hCD47-tg miniature swine for 2 days, and analyzed for hCD47 cross-dressing on gated PAOC47^{null} cells by FACS using anti-h/pCD47-PE mAb (the numbers in the figure indicate the MFI of gated PAOC/CD47^{null} cells). Two independent experiments were performed, and each experiment had 2 replicates per group. Representative FACS profiles are shown.

Cross-dressing with native CD47 from human T cell leukemia cells

We next determined whether cells can be cross dressed by native CD47 using human T-cell leukemia Jurkat cells that express a higher level of CD47 than normal hematopoietic cells (**Figure 2A**). In order to clearly identify cross-dressed CD47 on cell surface, CD47-deficient Jurkat cells were generated using the CRISPR-Cas9 technique (**Figure S3**) and cocultured for 24 h with the parental WT Jurkat cells or with hCD47-tg pig LCL cells (**Figure 2B**). FACS analysis revealed that CD47KO Jurkat cells were clearly stained positive by anti-hCD47 antibodies after coculture with parental WT Jurkat cells or hCD47-tg pig LCL cells compared to those cultured alone or mixed immediately before staining (**Figure 2B**). Furthermore, pig LCL cells also became positive for human CD47 staining after coculture for 24 h with WT Jurkat cells (**Figure 2B**). These results indicate that human CD47 cross-dressing could be induced by not only hCD47-transgenic cells but also tumor cells that express only the native CD47.

Figure S3. Generation of CD47-deficient Jurkat cells. (A) Schematic of human CD47 loci and guide RNA sequence targeting the exon 2 of human CD47 gene used for generating CD47KO Jurkat cells. (B) FACS profiles showing anti-CD47 staining of WT and CD47KO Jurkat cells.

Fig 2. CD47 expression and CD47 cross-dressing on human T-cell leukemia Jurkat cells. (A) CD47 expression on Jurkat cells and normal human CD3⁺, CD19⁺ and CD14⁺ peripheral blood cells (the numbers indicate MFI of human CD47 staining). (B) CD47 expression on WT Jurkat cells, pig LCL/CD47^{ph} cells, CD47KO Jurkat cells, CD47KO cells mixed with WT Jurkat cells (mixed at the time of staining), CD47KO Jurkat cells cocultured (24h) with WT Jurkat or pig LCL/CD47^{ph} cells, pig LCL cells, and LCL cells cocultured (24h) with WT Jurkat cells. The numbers in the figure indicate MFI of CD47 staining on gated CD47KO Jurkat cells and pig LCL cells.

Fig 3. CD47 cross-dressing by extracellular vesicles and exosomes. (A-B) CD47KO Jurkat cells (A) or pig LCL cells (B) were cultured in the absence (*left*) or presence (*right*) of EVs (*top*) or Exos (*bottom*) prepared from PAOC/CD47^{h2} cell culture supernatants for 2h or 6h, and analyzed for hCD47 cross-dressing by FACS using anti-hCD47-BV786 mAb. Representative FACS profiles of 3 independent

experiments were shown. (C) Celltrace violet labeled PAOC/CD47^{null} cells were cultured in the absence (*left*) or presence (*right*) of EVs prepared from PAOC/CD47^{h2} cells for 18h or 42h, and analyzed for hCD47 cross-dressing by FACS using anti-hCD47-BV786 mAb.

CD47 cross-dressing by extracellular vesicles and exosomes

We next investigated whether CD47 cross-dressing can be induced by extracellular vesicles (EVs) or requires direct cell-cell interaction. We analyzed hCD47 cross-dressing on CD47KO Jurkat cells, pig LCL cells and PAOC/CD47^{null} cells in the absence or presence of EVs prepared from PAOC/CD47^{h2} cells. FACS analysis showed that CD47 cross-dressing occurred in both CD47KO human T-cell leukemia Jurkat cells (**Figure 3A**) and pig B-lymphoma LCL cells (**Figure 3B**) after incubation for 2 or 6 hours with PAOC47^{h2} cell-derived EVs. To a less extent, both CD47KO Jurkat and LCL cells were also positively stained by anti-hCD47 after incubation with Exos released by PAOC47^{h2} cells (**Figure 3A,B**). Similarly, hCD47 cross-dressing was detected in PAOC^{null} cells after incubation with EVs from PAOC47^{h2} cells (**Figure 3C**). In this experiment, PAOC^{null} cells were labeled with fluorescence Celltrace violet prior to incubation with EVs to ensure there was no contamination by PAOC^{h2} cells in the prepared EVs. After incubation with PAOC47^{h2} EVs, a significant proportion of violet-labeled PAOC^{null} cells became positive for hCD47 (**Figure 3C**). Of note, the frequency of hCD47⁺ PAOC^{null} cells at 42 h was lower than that at 18h, which is most likely due to greater PAOC^{null} cell proliferation and EV exhaustion/degradation. These results indicate that CD47 cross-dressing could be induced independently of cell-cell contact by EVs, including Exos.

Protection against phagocytosis by cross-dressed CD47

We next determined whether cross-dressed CD47 can act as a marker of self to protect the cells against phagocytosis. We first investigated the binding potential of cross-dressed hCD47 with

human SIRP α . CD47KO Jurkat cells were cocultured without or with EVs from PAOC/CD47^{h2} cells for 5h, washed and incubated with recombinant human SIRP α -Fc chimera for 1h. Binding of human SIRP α fusion protein to CD47KO Jurkat cells was then measured using fluorochrome-labeled anti-human IgG Fc antibody. FACS analysis showed that human SIRP α fusion protein was able to bind CD47KO Jurkat cells cultured with EVs, but not those cultured without EVs (Figure 4A). The data indicate that cross-dressed hCD47 on CD47KO Jurkat cells can bind human SIRP α .

Fig 4. Protection against phagocytosis by cross-dressed CD47. (A) CD47KO Jurkat cells were cocultured without (*left*) or with (*right*) EVs from PAOC/CD47^{h2} cells at 37°C for 5h, then washed and incubated with recombinant human SIRP α -Fc chimera at 37°C for 1h. The binding of SIRP α -Fc proteins to CD47KO Jurkat cells was visualized by staining with APC-conjugated mouse anti-human IgG Fc mAb. Representative FACS profiles of 2 independent experiments are shown. (B) PAOC47^{null} and PAOC47^{h2} cells were cultured alone (*left* and *middle*) or together (*right*) for 48h, and stained using anti hCD47-BV786 mAb, then PAOC47^{null} (R1), PAOC47^{h2} (R3), and hCD47⁺ (i.e., hCD47 cross-dressed) PAOC47^{null} (R2) cells sorted from cocultures were used immediately for phagocytic assay. (C) PAOC47^{null} (R1), PAOC47^{h2} (R3), or sorted hCD47 cross-dressed PAOC47^{null} (R2) cells were labeled with Celltrace violet, and incubated with human macrophages for 2 h, then phagocytosis was determined

by FACS using anti-human CD14 mAb. Shown are representative FACS profiles (left) and levels (right; mean $\pm$ SDs; n = 3) of phagocytosis (i.e., percentages of human macrophages that have engulfed violet+ target cells (CD14⁺violet⁺) in human CD14⁺ macrophages). Representative results of 3 independent experiments are shown. (D) CD47KO Jurkat, WT Jurkat and CD47KO Jurkat cells pre-incubated with EVs from PAOC/CD47^{h2} cells were labeled by Celltrace violet, and cocultured with human macrophages for 2h, then phagocytosis was analyzed by FACS. Shown are representative FACS profiles (left) and levels (right; mean $\pm$ SDs; n = 3) of phagocytosis (i.e., percentages of hCD14⁺violet⁺ in total hCD14⁺ macrophages). Representative results of 2 independent experiments are shown. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, not significant (two-tailed unpaired t-test).

We then performed phagocytic assay to determine the potential of cross-dressed hCD47 to protect pig cells or CD47KO human leukemia cells against phagocytosis by human monocyte-derived macrophages. PAOC47^{null} and PAOC/CD47^{h2} cells were cultured for 48 h, then hCD47 cross-dressed PAOC47^{null} cells were sorted out (**Figure 4B**) and their susceptibility to phagocytosis by human macrophages was determined in comparison to PAOC47^{null} and PAOC/CD47^{h2} cells that were cultured separately (**Figure 4C**). As expected, PAOC47^{null} cells were significantly more sensitive than PAOC/CD47^{h2} cells to phagocytosis (**Figure 4C**). However, hCD47 cross-dressing effectively reduced the susceptibility of PAOC47^{null} cells to phagocytosis by human macrophages, to a level comparable to that of PAOC/CD47^{h2} cells (**Figure 4C**). Human CD47 cross-dressing protects not only xenogeneic pig cells, but also human leukemia cells, against phagocytosis by human macrophages. In phagocytic assays where CD47KO Jurkat cells showed significantly greater phagocytosis than WT Jurkat cells, pre-incubation of CD47KO Jurkat cells with EVs released by PAOC/CD47^{h2} cells was found highly effective in reducing their phagocytosis by human macrophages (**Figure 4D**). These results indicate that human CD47 cross-dressing can act as a functional ligand for human SIRP α and deliver “don’t eat me” signals to human macrophages.

Ligation of autogenous but not cross-dressed CD47 induces death in Jurkat cells

Ligation of cell surface CD47 by its ligand thrombospondin-1 (TSP-1) (Saumet et al., 2005), CD47-binding peptides of TSP-1 (Martinez-Torres et al., 2015) or CD47 antibodies (Mateo et al., 1999) has been shown to induce death in varying types of cells. In line with these reports, we observed that human SIRP α -Fc fusion proteins could induce cell death in a dose-dependent manner in WT, but not CD47KO, human T-cell leukemia Jurkat cells (**Figure S4; Figure 5A,B**). The cell death observed in WT Jurkat cells was induced by CD47 ligation with hSIRP α -Fc proteins, as cell death was minimally detectable in WT Jurkat cells that were cultured simultaneously without hSIRP α -Fc proteins. To determine whether cross-dressed CD47 on Jurkat cells may also induce cell death, we compared the susceptibility to cell death induced by SIRP α -Fc proteins among WT, CD47KO and hCD47 cross-dressed CD47KO Jurkat cells. CD47 cross-dressing was performed on GFP⁺ CD47KO Jurkat cells by incubation for 2h with PAOC/CD47^{h2} EVs. To induce cell death, GFP⁺ CD47KO or hCD47 cross-dressed GFP⁺ CD47KO Jurkat cells were cocultured, respectively, with an equal number of control WT Jurkat cells (5x10⁴ each) in the presence of 50nM human SIRP α -Fc for 1h. The cocultured cells were then stained with anti-hCD47 (BV786) mAb, and cell death in WT (hCD47⁺GFP⁻), CD47KO (CD47⁻GFP⁺), and hCD47 cross-dressed CD47KO (CD47^{low}GFP⁺) Jurkat cells were measured. Of note, binding of hSIRP α -Fc proteins to cell surface CD47 (either native or cross-dressed) could partially block subsequent staining with anti-hCD47 antibodies and thus, the cells cultured with hSIRP α -Fc showed relatively lower hCD47 staining than those cultured without (**Figure S5, Figure 5**). We found that SIRP α -Fc proteins induced significant cell death in WT Jurkat cells regardless of whether they were cocultured with CD47KO (**Figure 5C,D**) or with hCD47 cross-dressed CD47KO (**Figure 5E,F**). CD47 cross-dressing did not increase the sensitivity to cell death induced by SIRP α -Fc proteins, and cell death was minimally detectable in both CD47KO

(Figure 5C,D) and hCD47 cross-dressed CD47KO (Figure 5E,F) Jurkat cells. These results indicate that, unlike autogenous CD47, cross-dressed CD47 on Jurkat cells does not induce cell death upon ligation with SIRP α -Fc proteins.

Figure S4. SIRP α -Fc-induced cell death in WT and CD47KO Jurkat cells. 1×10^5 CD47KO or WT Jurkat cells were incubated in with hSIRP α -Fc proteins at the indicated concentrations for 1 h at 37°C, then stained with PI to identify dead cells. (A) Representative FACS profiles. (B) Percentages of dead (PI⁺) cells. Representative results of 2 independent experiments are shown.

Figure S5. Prior incubation with hSIRP α -Fc proteins partially blocks subsequent staining by anti-hCD47 mAb. (A) Anti-hCD47 staining of WT Jurkat cells with or without prior incubation with hSIRP α -Fc. (B) Anti-hCD47 staining of CD47KO Jurkat cells with or without prior incubation with hSIRP α -Fc. (C) Anti-hCD47 staining of PAOC/CD47^{h2} EV-treated (for 2h) CD47KO Jurkat cells with or without prior incubation with hSIRP α -Fc. The numbers in the figures indicate MFI levels. Results shown are representative of 3 independent experiments.

Fig 5. Ligation of autogenous but not cross-dressed CD47 induces cell death. (A-B) 5×10^4 WT Jurkat cells (GFP⁺) were incubated in the presence (A) or absence (B) of 50nM hSIRP α -Fc proteins at 37°C for 1h, and stained with anti-hCD47 mAb and PI. Representative FACS profiles show dead cells (PI⁺) in hCD47⁺ WT Jurkat population. (C-D) CD47KO Jurkat (GFP⁺) and WT Jurkat cells were mixed (at 1:1 ratio; 5×10^4 each) and cocultured in the presence of 50nM human SIRP α -Fc for 1h, then stained with anti-hCD47 mAb and PI. Shown are representative FACS profiles (C) and percentages (D; mean $\pm$ SDs) of PI⁺ dead cells in gated CD47KO (R1, CD47⁺GFP⁺) and WT (R2, hCD47⁺GFP⁺) Jurkat cells. (E-F) CD47KO Jurkat (GFP⁺) cells were incubated for 2h with PAOC/CD47^{h2} EVs, then mixed with WT Jurkat cells (at 1:1 ratio; 5×10^4 each) and cocultured in the presence of 50nM human SIRP α -Fc for 1h. The cells were stained with anti-hCD47 mAb and PI. Shown are representative FACS profiles (E) and percentages (F; mean $\pm$ SDs) of PI⁺ dead cells in gated EV-treated CD47KO (R1, CD47^{low}GFP⁺) and WT (R2, hCD47⁺GFP⁺) Jurkat cells. ****, $p < 0.0001$ (two-tailed unpaired t-test). Of note, the cells cultured with hSIRP α -Fc proteins (A, C and E) showed reduced hCD47 staining (as detailed in Figure S5).

Discussion

Given its strong inhibitory effects on macrophage activation and phagocytosis, transgenically overexpressed CD47 is considered an effective means of preventing transplant rejection. A major obstacle impeding the translation of xenotransplantation into clinical therapies is vigorous xenograft rejection (Yang and Sykes, 2007), and the lack of functional interaction in CD47-SIRP α pathway is a key mechanism triggering macrophage xenoimmune responses (Yang, 2010). Studies have shown that the use of gene-edited pigs carrying human CD47 is effective in protecting against xenograft rejection by macrophages in non-human primates (Ide et al., 2007, Tena et al., 2017, Watanabe et al., 2020). Recently, transgenic overexpression of CD47 was also successfully used in combination with other approaches, such as deletion of HLA molecules, to generate hypoimmunogenic pluripotent stem cells (Han et al., 2019, Deuse et al., 2019). Here we found that EVs and Exos released from pig cells transgenically overexpressing hCD47 can mediate hCD47 cross-dressing on surrounding pig cells. Furthermore, hCD47 cross-dressed on pig cells can interact with human SIRP α and inhibit phagocytosis by human macrophages. Such CD47 cross-dressing occurs not only in the pig-to-pig combination, but also in the human-to-human, pig-to-human, and human-to-pig directions. These results provide a new mechanism for the inhibition of phagocytosis by the approach of transgenically expressing CD47.

In both xenogeneic and allogeneic settings, a high level of transgenic CD47 expression was found to be essential for its immune inhibitory effect. CD47 is not only a ligand of SIRP α , but also a signaling receptor that mediates a variety of functions, including apoptosis, cell cycle arrest and senescence. It was reported that deletion of CD47 improves survival, proliferation and function of endothelial cells, leading to increased angiogenesis and neovascularization both *in*

vitro and *in vivo* (Meijles et al., 2017, Gao et al., 2016, Gao et al., 2017). In line with these observations, CD47 deletion from organ grafts was reported to ameliorate renal ischemia/reperfusion injury (Isenberg and Roberts, 2019) and cardiac allograft rejection (Chen et al., 2019). Using a pig-to-baboon kidney xenotransplantation model, a recent study suggested that widespread expression of hCD47 in the pig kidney was associated with increased vascular permeability and systemic edema, presumably due to upregulated TSP-1 and hence TSP-1-CD47 signaling in the graft (Takeuchi et al., 2021). Our study showed that, although CD47 cross-dressed on cells may bind CD47 ligands, the ligand engagement does not induce CD47 signaling to cause apoptosis. We found that ligation of hSIRP α -Fc proteins with autogenous, but not cross-dressed, CD47 on Jurkat cells induces cell death. This study suggests that using a pig vascularized organ with hCD47 overexpression in some cells, which are more sensitive to macrophage attack but relatively resistant to CD47 signaling-induced deleterious effects, may improve the outcomes of xenotransplantation.

Emerging evidence indicates that CD47-SIRP α signaling plays an important role in regulating DC activation and hence T cell priming. The initial evidence for a role of CD47 in controlling DC activation was obtained in a mouse model of DST, in which DST using WT cells induces donor-specific tolerance, but DST using CD47-deficient cells paradoxically induces SIRP α ⁺ DC activation and augments anti-donor T cell responses (Wang et al., 2010). A similar finding was made in a mouse model of hepatocyte allotransplantation where WT hepatocytes promote allograft survival, but CD47KO hepatocytes exacerbated rejection (Zhang et al., 2016). Although it was not tested directly, it is conceivable that CD47 cross-dressed on cells, which induces sufficient SIRP α signaling to inhibit macrophages, may suppress SIRP α ⁺ DC activation. Thus, in

addition to inhibition of phagocytosis, CD47 cross-dressing may also attenuate anti-donor T cell responses when transplants are performed using CD47-overexpressing donors.

CD47-SIRP α signaling is an important component of the tumor immune microenvironment. CD47 upregulation was found in many types of cancer cells, in which CD47 provides as important mechanism to evade macrophage killing (Jaiswal et al., 2009, Chan et al., 2009). CD47 upregulation in tumors also significantly contributes to tumor-induced T cell suppression, as the antitumor activity of CD47 blocking treatment is associated with CD11c⁺ DC activation and is largely T cell-dependent (Liu et al., 2015b, Chen et al., 2020, Li et al., 2020). In the present study, CD47 cross-dressing was found in human T-cell leukemia Jurkat and pig B-lymphoma LCL cells, suggesting a possible involvement of CD47 cross-dressing in the formation of a tumor immunosuppressive microenvironment. In addition, CD47 on EVs and CD47 cross-dressed on tumor cells may also neutralize CD47 ligands, such as TSP-1 that has been shown to induce CD47 activation leading to apoptosis in tumor and endothelial cells (Martinez-Torres et al., 2015), hence favoring tumor growth.

While the mechanisms of EV-mediated exchange of biological information and materials between cells remains poorly understood (Raposo and Stahl, 2019), EV-induced antigen cross-dressing has been reported to play an important role in regulation of immune responses, including alloantigen recognition and allograft rejection (Zeng and Morelli, 2018, Gonzalez-Nolasco et al., 2018). Earlier studies have shown that CD47, as a “don’t eat me” signal, is essential for EVs to elicit biological function by preventing their clearance by macrophages (Kamerkar et al., 2017). Here we report that EV-induced CD47 cross-dressing possesses partial

activity of the autogenous CD47, such as the ability to initiate inhibitory SIRP α signaling, and therefore offers means of separating the desired and harmful effects of CD47.

Materials and methods

Cell culture

WT Jurkat cell line (J.RT3-T3.5, ATCC® TIB-153™) was purchased from ATCC. Pig aortic endothelial cells (PAOC) immortalized with SV40 were purchased from ABM (catalog # T0448). Human CD47 (hCD47) transgenic (tg) pig B-lymphoma cell line (LCL) cell line (hCD47-tg LCL) and control pig B LCL cell line were generated by transfecting porcine B LCL cells with pKS336-hCD47 or empty pKS336 vector, respectively, as described previously (Ide et al., 2007). Human CD47-tg porcine bone marrow cells (BMCs) were harvested from SLA-defined miniature swine with hCD47 transgene (Watanabe et al., 2020). All PAOC cell lines were grown in Endothelial Cell Growth Medium (Cell Applications) supplemented with 10% FBS (Atlanta Biologicals). All other cell lines were grown in Dulbecco's Modified Eagle's Medium (Gibco) + GlutaMax (Thermo fisher scientific) supplemented with 10% FBS (Atlanta Biologicals) and 100 U/ml penicillin and streptomycin (Gibco).

Generation of CD47KO Jurkat and PAOC sublines

CRISPR small guide RNA (sgRNA) for disrupting hCD47 in Jurkat cells was designed using the online tools (<https://crispr.mit.edu>), with sequence targeting the exon 2 of hCD47 (CTACTGAAGTATACGTAAAG-TGG (PAM)). The sgRNA was cloned into the pL-CRISPR.EFS.GFP lentiviral vector which was a gift from Benjamin Ebert (Addgene plasmid # 57818) for co-expression with Cas9 (Heckl et al., 2014). Lentiviral particles were produced by

co-transfection of a 3-plasmid system consisting of the pL-CRISPR.EFS.GFP vector and packaging plasmids (pVSV-G and p Δ) using CaCl₂ into 293T cells in 175cm² flasks. Lentivirus supernatant was collected 48h post-transfection, concentrated by ultracentrifugation at 22,000 rpm for 2.5 hours (Beckman Coulter, Optima XE-90) and stored at -80°C until use. GFP⁺ cells were sorted 3 days after lentivirus transduction, then sorted GFP⁺ cells were expanded and assessed for CD47 expression by staining with BV786-conjugated anti-hCD47 mAb B6H12 (BD Bioscience) and PE-conjugated anti-hCD47 mAb CC2C6 (Biolegend). CD47 negative Jurkat cells were established by 4 rounds of cell sorting.

PAOC cells were immortalized with Lenti-hTERT virus (ABM; cat# G200) following manufacturer's instructions and clonal sorting/expansion. Alpha GAL KO pAOC-SV40-hTERT (GTKO) cell line (PAOC/CD47^p) was created via nucleofection of pAOC-SV40-hTERT with plasmid expressing Cas9 protein (GeneART CRISPR Nuclease Vector, Invitrogen) and guide RNA targeting GGTA-1 gene (aGal protein, guide RNA sequence used:

TCATGGTGGATGATATCTCC) using Lonza 4D-Nucleofector, followed by clonal sorting/expansion. PAOC/CD47^{null} was created via nucleofection of PAOC/CD47^p cells with plasmid expressing Cas9 protein and guide RNA targeting the pig CD47 gene (guide RNA sequence used: TCACCATCAGAATTACTACA) using Lonza 4D-Nucleofector.

PAOC/CD47^{null} and PAOC/CD47^p cell lines expressing hCD47short (305aa, 16aa short intracellular domain, NM_198793, NP_942088, PAOC/CD47^{h2} and PAOC/CD47^{p/h2}) or hCD47long isoforms (323aa, 34aa long intracellular domain, NM_001777, NP_001768, PAOC/CD47^{h4} and PAOC/CD47^{p/h4}) (Reinhold et al., 1995) were created via nucleofection with plasmid expressing Cas9 protein (GeneART CRISPR Nuclease Vector, Invitrogen) and guide

RNA targeting AAVS1 safe harbor site using Lonza 4D-Nucleofector, followed by clonal sorting/expansion.

Flow cytometric analysis

CD47 expression or cross-dressing on cells was determined by direct staining with BV786-conjugated anti human specific CD47 mAb B6H12 (abbreviated anti-hCD47; BD Bioscience) or PE-conjugated anti-human CD47 mAb CC2C6 (with cross-reactivity to pig CD47 and thus, referred to as anti-h/pCD47; Biolegend). The level of cell surface CD47 is expressed as median fluorescent intensity (MFI). FITC-conjugated anti-pig SIRP α mAb (clone BL1H7) was from Abcam. Recombinant human SIRP α /CD172a Fc chimera protein, CF (cat# 4546-SA-050) was from R&D system. APC-conjugated anti-human IgG Fc (clone HP6017) was from Biolegend. For analysis of CD47 expression on human PBMCs, single cell suspensions were incubated with anti-hCD47 mAb B6H12 in combination with fluorochrome-conjugated anti-human CD45 (clone HI30), CD19 (clone HIB19), CD3 (clone SK7), and CD14 (clone M5E2; all from Biolegend). Dead cells were identified by staining with propidium iodide. All samples were collected on FACS Flow Cytometer (Fortessa, Becton Dickinson) and data were analyzed by Flowjo software (Tree Star).

Purification of extracellular vesicles

Extracellular vesicles (EVs) and exosomes (Exos) from cell culture supernatants were purified by a standard differential centrifugation protocol as previously reported (Chen et al., 2018). In brief, bovine exosomes were depleted from FBS by overnight centrifugation at 100,000g, PAOC/CD47^{h2} cells were cultured in media supplemented with 10% exosome depleted FBS for

EVs and Exos purification from cell culture supernatants. Supernatants collected from 48 h cell cultures were centrifuged at 2,000g (3,000rpm) for 20 min to remove cell debris and dead cells. Extracellular vesicles were pelleted after centrifugation at 16,500g (9,800rpm) for 45 min (Beckman Coulter, Optima XE-90) and resuspended in PBS. The pelleted exosomes from above supernatants were further centrifuged at 100,000g (26,450rpm) for 2 h at 4 °C (Beckman Coulter, Optima XE-90) and resuspended in PBS. EVs and Exos from total 1.3×10^7 PAOC/CD47^{h2} cells cultured for 48 h were concentrated in 250ul and 400ul PBS respectively, 10ul of each was used for CD47 cross-dressing.

Preparation of human macrophages

Blood from healthy volunteers was used to prepare peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation. PBMCs were added at 3×10^6 per well in a 24-well plate and unattached cells were removed from the plate on the second day. Attached cells were then differentiated to macrophages by 8–9 d of culture in IMDM (Gibco)+ GlutaMax (Thermo fisher scientific) supplemented with 10% AB human serum (Gemini Bio-products, Inc.) containing 10ng/ml human M-CSF (PeproTech) and 100 U/ml penicillin and streptomycin (Gibco). The use of human blood samples was approved by the Institutional Review Board of Columbia University Medical Center.

Flow cytometry-based phagocytic assay

Macrophages generated as above were harvested from plates using Trypsin-EDTA (Thermo fisher scientific). The indicated target cells were labeled with Celltrace violet (Thermo fisher scientific) according to the manufacturer's protocol, and phagocytic assay was performed by co-

culturing 6×10^4 Celltrace Violet-labeled target cells with 3×10^4 human macrophages for 2h in ultra-low attachment 96-well flat bottom plates in IMDM + GlutaMax without antibiotics or serum added. All cells were harvested after co-culture and phagocytosis was determined by FACS analyses, in which the phagocytic ratio is calculated as the percentage of macrophages that engulfed target cells (human $CD45^+CD14^+$ Celltrace violet⁺) among total macrophages (human $CD45^+CD14^+$).

Statistical analysis

Data were analyzed using GraphPad Prism (version 8; San Diego, CA) and presented as mean value $\pm$ SDs. The level of significant differences in group means was assessed by student's t-test, and a p value of ≤ 0.05 was considered significant in all analyses herein.

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Author Contributions

Y.L., Y.W., E.A.F., X.W. A. D., and X.H. performed experiments; Y.L., Y.W., X.W., H.W., R.J.H., S.S., M.S., and Y-G.Y. discussed the project, designed experiments and/or analyzed data; Y-G.Y. conceived the research project and directed the research; Y.L. and Y-G.Y. wrote the paper; all authors edited and approved the paper.

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506 **Declaration of interests**

507 The authors declare no competing interests.

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