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Transgenic expression of human CD47 reduces phagocytosis of porcine endothelial cells and podocytes by baboon and human macrophages

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Abstract

Our initial studies utilizing a 1,3-galactosyltransferase (GalTKO) knockout pig-to-baboon renal transplant model demonstrated that the early development of nephrotic syndrome has been a significant obstacle to the long-term survival of baboon recipients. We have recently documented that sphingomyelin phosphodiesterase-3 (SMPDL3b) and CD80 expressed on podocytes in porcine kidney grafts contribute to this complication. We have hypothesized that one regulator of immune function is CD47, and that incompatibilities in CD47 between pig and baboon could potentially affect macrophage function, increasing the susceptibility of the kidney grafts to immunologically-induced injury. In order to address this hypothesis in vitro, we isolated and cultured porcine podocytes and ECs from GalTKO alone, human CD47 (hCD47)/hCD55-expressing transgenic (Tg) GalTKO swine, as well as GalTKO hCD46/hCD55-Tg swine along with baboon or human macrophages. We found that baboon macrophages phagocytosed porcine ECs in a similar manner to human macrophages, and this response was significantly reduced when porcine ECs and podocytes expressed hCD47/hCD55 but not hCD46/hCD55 without hCD47. Furthermore, masking hCD47 by anti-hCD47 antibody on hCD47/hCD55Tg ECs restored

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Shunichiro Nomura: Primarily performed in vitro experiments and participated in writing the manuscript.

Yuichi Ariyoshi, Hironosuke Watanabe, Kazuhiro Takeuchi and Masayuki Tasaki: Participating in in vitro assays.

Thomas Pomposelli, Gabriela Garcia, and Richard Johnson: Participated in discussion and writing the manuscript.

David Ayares: Provided Tg pig and participated in discussion

Megan Sykes, David H. Sachs: Participated in discussion and data analysis

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by *Xenotransplantation*.

phagocytosis. These results are consistent with the hypothesis that CD47 incompatibility plays an important role in promoting macrophage phagocytosis of endogenous cells from the transplanted kidney. The similar levels of phagocytosis of porcine cells by baboon and human macrophages suggest that the expression of hCD47Tg on glomerular cells in donor porcine kidneys may prove to be a key strategy for preventing proteinuria following kidney xenotransplantation in a pig-to-human as well as a pig-to-baboon model.

Keywords

Xenotransplantation; CD47 Tg; Proteinuria; Podocytes; Endothelial Cells; Pig-to-Baboon/Human

INTRODUCTION

Our initial study of a life-supporting kidney from a GalTKO pig to baboon xenotransplant model with co-transplantation of a vascularized donor thymus (XKTx+VT) in 2003 achieved survival for 83 days¹. Despite immunologic unresponsiveness in cellular and anti-pig antibody (ab) responses *in vitro*, baboons uniformly developed proteinuria as early as post-operative day (POD) 2, which limited the average survival of baboon recipients of XKTx+VT to 2 months²³. Similar to clinical nephrotic syndrome, the proteinuria in our model induced ascites and pleural effusions which made the animals prone to infection and cardiorespiratory failure⁴⁵⁶⁷.

In order to resolve this major obstacle, we have sought to elucidate the causes of early proteinuria in our XKTx+VT model. Our recent data indicated that sphingomyelin phosphodiesterase-3 (SMPDL3b) and upregulation of CD80 expression on podocytes in porcine kidney grafts in nephrotic baboons were likely contributors³⁸. We hypothesized that a regimen which includes the anti-CD20 monoclonal antibody (mAb) rituximab might prevent proteinuria, as it is known to bind to SMPDL3b on porcine podocytes. In addition, similar to clinical minimal change disease (MCD), CD80 on glomerular podocytes was upregulated following XKTx+VT, without histologic and immunologic evidence of rejection⁸. By adding rituximab at the time of revascularization of porcine kidney grafts to mask SMPDL3b³ as well as a weekly dose of CTLA4-Ig, we were able to reduce proteinuria and improve survival of a recipient of a GalTKO thymokidney for up to 193 days⁸. This is thus far the longest functioning porcine kidney graft without additional transgenic (Tg) modification in a pig-to-baboon XKTx model.

Recent progress in gene editing technology has facilitated the survival of life-supporting solid organ xenografts from a couple of months, to several months, to up to a year in pig-to-baboon models⁸⁹¹⁰. Although multiple Tgs can be added, it is now important to determine what genes should be added to potential source pigs for clinical XTtx. Furthermore, our data with a tolerance-inducing regimen involving donor VT grafts suggest that a sequential cascade associated with SMPDL3b and CD80 upregulation are the leading factors that cause the observed podocytosis³⁸.

In this study, we focused on CD47, which is known to be a species-specific immune inhibitory receptor on macrophages^{11, 12}. We specifically asked whether (1) species

incompatibility induces robust phagocytosis via this pathway, involving mainly endothelial cells (ECs) and podocytes; (2) if EC and podocyte phagocytosis by baboon macrophages is associated with CD47 incompatibility and if this response similarly occurs in human macrophages, (3) if transgenic expression of human CD47 (hCD47) inhibits this phagocytosis; and (4) whether masking pig native CD47 would increase or decrease phagocytosis.

Materials and Methods

Animals that were used for isolation of porcine podocytes, ECs and macrophages

Pigs: We used two different strains of GalTKO pigs. One was the MHC inbred GalTKO miniature swine with or without genetic modification with hCD47 and hCD55¹³¹⁴. The other was outbred domestic swine provided by Revivicor Inc. (Blacksburg, VA) that carried hCD46/hCD55/hCD39/hCD141/hCD201 genetically modified GalTKO. As a control, we used podocytes or ECs from a Gal positive MHC inbred miniature swine or domestic swine¹⁵.

Baboons: *Papio hamadryas* were purchased from the Mannheimer Foundation (Homestead, FL).

Porcine Podocyte Isolation and Culture

Right naïve kidneys were used to isolate podocytes. Right nephrectomy was performed at the time of left nephrectomy for XKTx. The porcine podocyte cultures were developed using a method that we have previously reported³. Expression of hCD47 on podocytes was confirmed on the basis of staining with anti-nephrin (Santa Cruz Biotechnology, Inc.) and anti-human CD47 (Abcam, Cambridge, MA) ab assessed by immunofluorescence microscopy as well as flow cytometric analysis (FCM. FACS CANTO II, BD Biosciences, CA) and Reverse Transcription Polymerase chain reaction (RT-PCR) (see below). In order to assess hCD47 expression by FCM anti-human CD47 mAbs (B6H12) (BioLegend, San Diego, CA) was used.

Porcine Endothelial Isolation and Culture

Porcine aortas were harvested in a single segment from thoracic to abdominal compartments from donors of XKTx in order to isolate porcine aortic endothelial (PAECs) cells. In order to isolate PAECs, the excised aortas were incubated with collagenase III (2.5%; Sigma-Aldrich, Lyon, France) for 30 min at 37 °C. After digestion, cells were harvested and after two washes, PAECs were seeded on culture dishes pre-coated with 1% gelatin (Sigma-Aldrich). PAECs were grown in Medium 199 supplemented with 10% fetal bovine serum, glutamine (200 mM), Penicillin and streptomycin (100 UI/ml) at 37 °C in a 5% CO₂. PAECs were used between passages 2 and 6. The cells were confirmed to be hCD47 Tg PAECs on the basis of staining with anti-CD31 (Abcam) and anti-human CD47 (Abcam, Cambridge, MA) mAb assessed by immunofluorescence microscopy as well as FCM and RT-PCR.

RT-PCR Analysis of hCD47

Total RNA from podocyte and ECs was isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany). The quantity and quality of RNA were evaluated by spectrophotometry. Reverse transcription of RNA to cDNA was achieved using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Polymerase chain reaction (PCR) mixture was prepared using Dream Taq green PCR Master Mix (Thermo Fisher Scientific Inc., Waltham, MA). The primers used are human CD47 (Hs00177953_m1) and GAPDH (Hs02786624_g1) as gene-specific probes (Applied Biosystems). Thermal cycling conditions were 1 minute at 95°C, followed by 40 cycles of 95°C for 30 seconds, and 55°C for 30 seconds, using ABI 7500 System (Applied Biosystems, Foster City, CA, USA). Amplified PCR products were analyzed by agarose gel (2%) containing ethidium bromide.

Isolation of Baboon and Human Macrophages

Macrophages were obtained using a published protocol^{16–18}. Briefly, a total of 5×10^7 human or baboon Peripheral Blood Mononuclear Cells (PBMCs) were plated on a 25 cm² cell culture flask (Corning) with 5 mL of RPMI1640 containing 10% Fetal Bovine Sera (FBS) and incubated for 2 hours. The supernatant with non-adherent cells was then removed and washed with PBS. Human recombinant macrophage colony-stimulating factor (Sigma-Aldrich) (50 ng/mL in 5 mL of culture medium) was added and the cells were cultured for 7 days. The culture media was replaced every 3 days. Seven days after culturing, the medium was removed and the adherent cells were washed with PBS. Subsequently, 1 mL of 0.25% Trypsin/EDTA solution was added and the suspension, which was then incubated for 30 min at room temperature with gentle tipping of the dish to dissociate the macrophages. The cell suspension was centrifuged at 1000 rpm for 5 min.

The purity of CD14 macrophages was confirmed by FCM analysis immunofluorescence by FACS CANTO II (BD Biosciences, CA). More than 95% of the cells demonstrated positivity for the CD14 antigen. Without any pre-culture, freshly isolated human or baboon macrophages were immediately subjected to phagocytosis assays.

Immunocytochemistry and FCM Analysis of hCD47 on isolated ECs and podocytes

In order to confirm successful isolation of PAECs and podocytes and determine hCD47 expression, cells were assessed by both immunocytochemistry and FCM. For the immunocytochemistry analysis, isolated cells from porcine aorta and porcine kidneys were seeded at approximately 60% confluence in glass bottom culture dishes. Cells were rinsed with PBS and fixed with freshly prepared with 4% PFA (Fisher Scientific) for hCD47 or methanol for nephrin and CD31. Cells were incubated for 1 hr at 20 °C with anti-human CD47 ab (Abcam). After rinsing with PBS, cells were incubated with a fluorescein horse anti-mouse IgG ab (Vector) for 1 hr then briefly incubated with DAPI. Isolated porcine aortic cells and renal cells were also assessed by FCM with anti-human CD47, anti-CD31 mAb for ECs and anti-human CD47 and nephrin for podocytes. Nephrin was stained using an intracellular staining kit (eBioscience).

Phagocytosis Assays

Target cells were stained with the fluorescent dyes 5/6-CFSE (Molecular Probes, Eugene, OR) according to the manufacturer's protocol. CFSE-labeled target cells (1×10^5) were incubated with human and baboon macrophages (1×10^5) in 24-well flat-bottom plates (Costar, Corning, NY). The macrophages engulfing target porcine cells could be identified as CFSE-labeling cells by FCM analyses. The cells were harvested at the indicated times and stained with allophycocyanin-conjugated mouse anti-human CD14 ab (M5E2) (BioLegend) before FCM analysis. Blocking assays were performed using anti-human CD47 ab (BioLegend), which has been shown not to cross react with pig cells, as well as anti-pig ab (Antigenix America, NY) that cross-reacts to human cells. hCD47 Tg GalTKO ECs were pre-incubated with either the anti-human CD47 ab or control IgG ab, and GalTKO without hCD47 Tg ECs were preincubated with the anti-pig CD47 ab at $10\mu\text{g ml}^{-1}$ for 2hrs before being co-cultured with macrophages. Assays were performed at least in triplicate and repeated on at least three different days using different macrophage donors.

Statistical Analysis

Statistical analysis of the differences between two groups were assessed by t-test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Confirmed hCD47 expression on isolated porcine ECs and podocytes from hCD47 Tg pigs

Expression of hCD47 on isolated ECs and podocytes from kidneys of hCD47 Tg pigs were confirmed by FCM, RT-PCR and immunofluorescence microscopy, (Figs. 1. 2). Immunofluorescence staining showed that isolated cells from hCD47 Tg pig aortae were CD31 positive (Fig 1A right) and expression of hCD47 was seen on their cell surfaces (Fig. 1A left). FCM (Fig. 1B) and RT-PCR (Fig. 1C) also confirmed that only isolated ECs from hCD47 Tg pigs were hCD47 positive. Isolated renal cells were spindle-shaped and nephrin positive, indicating that they were podocytes (Fig 2A left), and those from hCD47 Tg pigs were hCD47 positive on the cell surface (Fig 2A right). Similar to ECs, FCM (Fig. 2B) and RT-PCR (Fig. 2C) confirmed that podocytes from hCD47 kidneys were hCD47 positive while neither podocytes from Gal positive (Gal+) miniature swine nor GalTKO miniature swine were hCD47 positive. FCM data demonstrated that >99% of cells from aortae of hCD47 Tg pigs were CD31+/hCD47+ (Fig. 1B right), and >99% of isolated renal cells were nephrin+/hCD47+ (Fig. 2B right). These cells were used for co-culture assays to assess the phagocytic activity of human or baboon macrophages.

Phagocytosis of porcine ECs by baboon macrophages is similar to that by human macrophages, and is reduced when ECs express hCD47

Isolated and expanded cells from baboons and humans were >98% CD14 positive. These cells were co-cultured with either PAECs (Fig. 3 and 4) or podocytes (Fig. 5). Phagocytosis was assessed by FCM using CFSE labeled ECs or podocytes and CD14 positive human or baboon macrophages. The macrophages that phagocytosed the target cells were identified as CD14 and CFSE double positive cells (Fig. 3–5). Cell fusion was excluded by a gating

strategy set up to detect cellular debris and doublets with forward and side scatter signals of area, width, and height.

Assays were performed in triplicate and repeated on three different days using different macrophage donors. Figure 3A shows representative FCM data of human CD14 cells co-cultured with various porcine ECs. 44.85%, 48.48% 47.79% or 51.96% of cells were double positive in human CD14+ cells with Gal+ domestic (Fig. 3A-1), Gal+ miniature swine (Fig. 3A-2), GalTKO without additional Tg (Fig. 3A-3) or GalTKO with Tg including hCD46 and hCD55 without hCD47 (Fig. 3A-4) ECs while only 22.53% were double positive in co-culture of human CD14+ cells with hCD47+/hCD55Tg ECs (Fig. 3A-5). We calculated (1) the percent (%) phagocytosis calculated by both % of human macrophages that phagocytized pig ECs (Fig. 3B left) and (2) % of pig ECs that were phagocytized by human macrophages (Fig. 3B right). A statistically significant reduction of phagocytosis was found when human CD14+ cells were co-cultured with hCD47+ EC (Fig. 3B #5 bars. Average % phagocytosis $\pm$ SD) as compared with Gal+ or GalTKO without hCD47Tg. GalTKO with hCD55Tg without hCD47 Tg showed no reduction of phagocytosis (Fig. 3B #4 bars).

A similar pattern was observed when baboon macrophages were used instead of human macrophages. 45.35%, 43.55%, 41.41% or 38.72% of cells were double positive in baboon CD14+ cells with Gal+ domestic (Fig. 4A-1), Gal+ miniature swine (Fig. 4A-2), GalTKO without additional Tg (Fig. 4A-3) or GalTKO with Tg including hCD46 and hCD55 without hCD47 ECs (Fig. 4A-4) while only 18.82% were double positive in co-culture of baboon CD14+ cells with hCD47+/hCD55Tg ECs (Fig. 4A-5). % phagocytosis also showed statistically significant differences between hCD47+ and hCD47- ECs (Fig. 4B #5 bars vs #1, 2, 3 and 4 bars in 4B left and right)

Phagocytosis of porcine podocytes by baboon macrophages were significantly reduced in podocytes expressing hCD47

We also examined if hCD47 expression on porcine podocytes reduced phagocytosis by baboon macrophages. We observed a similar pattern to that seen with ECs. 50.36%, 46.97% or 44.39% of cells were double positive in baboon CD14+ cells with Gal+ domestic (Fig. 5A-1), Gal+ miniature swine (Fig. 5A-2), or GalTKO with Tg including hCD46 and hCD55 without hCD47 podocytes (Fig. 5A-3) while only 19.98% were double positive in co-culture of baboon CD14+ cells with hCD47+/hCD55Tg podocytes (Fig 5A-4). % phagocytosis also demonstrated statistically significant differences between hCD47+ and hCD47- podocytes (#4 bars vs #1, 2 and 3 bars in 5B left and right). Assays were performed in triplicate and repeated on three different days using different macrophage donors.

Masking hCD47 on hCD47+ ECs restored phagocytosis

We next performed masking of CD47 expression by either anti-human CD47 or anti-pig CD47 ab. Figure 6A and 6B show the FCM profile which assesses cross reactivity of human ab and anti-pig CD47 ab using GalTKO pig, baboon and human PBMC. Although the anti-human CD47 ab cross reacted to baboon PBMCs (Fig. 6A middle and right), no cross-reactivity was seen against pig PBMCs (Fig. 6A left). Anti-pig CD47 ab, however, bound to not only pig CD47 on GalTKO PBMC but also cross reacted with baboon and human PBMC

(Fig. 6B). Thus, we examined whether (1) masking hCD47 on hCD47Tg GalTKO ECs restores phagocytosis, and (2) masking pig CD47 on GalTKO ECs (without hCD47Tg) changes the percent of phagocytosis. Figure 6C and D show representative data. While hCD47 porcine ECs had only 17.85% of double positive hCD14 and CSFE baboon macrophages (Fig. 6C-1), phagocytosis was restored to 34.98% when hCD47 expression was masked by anti-human hCD47 ab (Fig. 6C-2). Assays were performed in quintuplicate and repeated on three different days using different macrophage donors. % of baboon macrophages that phagocytosed hCD47Tg pig ECs were 58.5 $\pm$ 1.7% with anti-human hCD47 ab and 39.4% with control IgG, and % of pig ECs that were phagocytosed by baboon macrophages were 51.9 $\pm$ 1.7% with anti-human hCD47 ab and 24.3% with control IgG. On the other hand, no marked change in phagocytosis of GalTKO ECs was seen when pig native CD47 was masked with anti-pig CD47 ab (Fig. 6D). % of baboon macrophages that phagocytosed pig GalTKO ECs were 59.9 $\pm$ 3.9% with anti-pig CD47 ab and 63.3% with isotype control. % of pig GalTKO ECs that were phagocytosed by baboon macrophages were 58.6 $\pm$ 2.6% with anti-pig hCD47 ab and 55.7% with isotype control.

DISCUSSION

We hypothesized that an incompatibility in CD47 between the host and the donor kidney after transplant might result in an enhanced immune response against the xenograft. To test this hypothesis, we evaluated the ability of baboon and human macrophages to phagocytose podocytes and endothelial cells from Gal+, GalTKO and hCD47Tg pigs, and also sought to determine if expressing human CD47 altered this phagocytosis. Our present study demonstrated that (1) species incompatibility between baboons and pigs induced endothelial and podocyte phagocytosis in a similar manner to that observed between humans and pigs, (2) hCD47 expression on porcine ECs or podocytes, but not hCD46 and/or hCD55, inhibits phagocytosis, and (3) preincubation of hCD47 Tg ECs with anti-human ab that cross-reacts with baboon CD47 but not to pig CD47 in co-culture assays restored phagocytosis, while incubation with anti-pig ab did not change phagocytosis of GalTKO ECs. This is the first report evaluating phagocytosis of ECs and podocytes that were isolated from hCD47 Tg GalTKO swine by baboon and human macrophages.

Recent gene editing technology now allows for the production of multiple Tgs quickly and effectively¹⁹²⁰. However, it is now important to determine what genes should be added to potential donors for clinical XTx. Although recent studies from other groups showed proteinuria that developed along with rejection, one model used multi-Tg pigs with multiple anti-inflammatory drugs⁹ and the other used rhesus macaques as recipients²¹. We, on the other hand, have observed proteinuria without rejection in baboons receiving kidneys and vascularized thymus transplants from GalTKO pigs without hCD47 Tg¹²³⁸. Importantly, our results have shown the similarity of the effect of hCD47 on macrophage-mediated phagocytosis between humans and baboons. Our preliminary data demonstrated expression of hCD47 Tg on glomerular cells in GalTKO pig kidneys prevented post XKTx proteinuria in our pig-to-baboon model (Nomura S and Yamada K et al. TTS 2018), suggesting hCD47 may play an important role in minimizing the risk of post pig-to-human XKTx proteinuria.

CD47 serves as a ligand for signal regulatory protein alpha (SIRP α ; CD172a, SHPS-1), which is an immune inhibitory receptor on macrophages²². CD47 and SIRP α constitute a cell-cell communication system (the CD47-SIRP system) and incompatibility of CD47-SIRP α plays a significant role in the negative regulation of phagocytosis by macrophages²³. Wang et al have reported that expression of mouse CD47 on porcine B lymphoma-like cells prevented phagocytosis of these cells by mouse macrophages²⁴. Others also demonstrated inhibition of phagocytosis of a porcine lymphoblastoid cell line by human macrophages in vitro¹¹. However, to our knowledge, this is the first report to examine the investigation of the role human and porcine CD47 expression by porcine glomerular podocytes and ECs in phagocytosis by baboon and human macrophages.

Human CD47 expression on porcine cells markedly reduced, but did not totally prevent phagocytosis from human macrophages. This may be due to the fact that not only inhibitory signals are needed, but that suppression of xenoantigen-induced activating signals may also be required to completely prevent the phagocytic activity of human macrophages on porcine cells¹¹. It is also possible that Thrombospondin 1 (TSP-1), which is produced by activated macrophages and is also a ligand of CD47, could competitively bind to hCD47 expressed on EC and podocytes to block CD47-SIRP α interaction. Since tissue factor pathway inhibitor (TFPI) binds to TSP-1, pig cells simultaneously expressing hCD47 and TFPI might more efficiently prevent macrophage-mediated phagocytosis as described²⁵. We are currently investigating this possible strategy both *in vitro* and *in vivo*.

Based upon our *in vitro* data in the current study, we are using hCD47 Tg GalTKO pigs as donors of life-supporting solid organ Tx in pig-to-baboon models. Our *in vivo* results in lung XTX demonstrate that the use of hCD47 Tg pigs that express hCD47 on ECs protected porcine grafts from early vascular injury, which resulted in lung graft survival increasing from an average of 3.75 days to 8.75 days²⁶. XKTx studies using hCD47Tg pigs are currently in progress. Preliminary data presented at TTS 2018 (Nomura and Yamada K et al.) showed that high glomerular expression of hCD47 in porcine kidneys minimized the development of proteinuria while trace expression of hCD47 did not prevent it (Yamada K et al. manuscript in preparation).

In conclusion, our data suggested that species CD47 incompatibility between pig donors and baboon/human recipients could lead to enhanced macrophage immune responses in porcine renal podocytes and ECs. It is possible this may play a role in both graft loss and the development of proteinuria that occurs commonly in the xenograft, and further studies are needed to investigate this possibility.

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Abbreviations:

EC	endothelial cells
GalTKO	galactosyl- α 1–3-galactosyl transferase gene knockout
PAEC	porcine aortic endothelial
PBMC	Peripheral Blood Mononuclear Cells
POD	post-operative day
RT-PCR	Reverse Transcription Polymerase chain reaction
Tg	transgenic
SIRP	signal regulatory protein
TK	thymokidney
hCD47	human CD47
TSP-1	Thrombospondin 1
TFPI	Tissue factor pathway inhibitor
XKTx+VT	kidney xenotransplant with co-transplant of a vascularized donor thymus

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Figure 1.

(A) Expression of human CD47 (hCD47) and pig CD31 (pCD31) on ECs from an hCD47 positive GalTKO MHC inbred miniature swine was confirmed by immunofluorescence microscopy. (B) hCD47 and pCD31 expression on ECs were confirmed by FCM. hCD47 positive GalTKO MHC inbred miniature swine (right) and isotype controls (left). (C) human CD47 Tg was confirmed by RT-PCR using a specific primer. Lane 1, ECs from a Gal+ MHC inbred miniature swine; Lane 2, ECs from a GalTKO MHC inbred miniature swine; Lane 3,

ECs form a hCD47 positive GalTKO MHC inbred miniature swine. GAPDH was used as a DNA loading control.

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Figure 2.

(A) Immunofluorescence staining of renal cells that were isolated from hCD47 positive GalTKO MHC inbred miniature swine. Expression of nephrin (A left) and human CD47 (A right) confirmed that these morphologically spindle-shape renal cells were hCD47+ podocytes. (B) human CD47 Tg podocytes was confirmed by double staining of hCD47 and nephrin in FCM. Similar to ECs cells hCD47 was positive on podocytes that were isolated from GalTKO hCD47Tg MHC inbred swine (B right) and isotype controls (left). These cells were also nephrin positive. (C) human CD47 Tg was confirmed by RT-PCR using a specific

primer. Lane 1, podocytes from a Gal⁺ MHC inbred miniature swine; Lane 2, podocytes from a GalTKO MHC inbred miniature swine; Lane 3, podocytes from a hCD47 positive GalTKO MHC inbred miniature swine. GAPDH was used as a DNA loading control.

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Figure 3.

Human CD47 positive porcine ECs attenuate phagocytosis by human macrophages. (A) Representative data of FCM profile. CFSE-labeled ECs from domestic swine, MHC inbred miniature swine, MHC inbred miniature GalTKO swine, hCD46/hCD55 positive GalTKO domestic swine, and hCD47/hCD55 positive GalTKO MHC inbred miniature swine were incubated with human macrophages for 4 hours at 37°C. Human macrophages counterstained with allophycocyanin-conjugated anti-human CD14 and phagocytosis of CFSE-labeled targets were measured by FCM analysis. 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: (B left) Percentages of human macrophages that phagocytized pig ECs = (percentage in upper right quadrant/ percentage in upper left quadrant + percentage in upper right quadrant) x 100. (B right) Percentages of pig ECs that were phagocytized by human macrophages = (percentage in upper right quadrant/ percentage in upper right quadrant + percentage in lower right quadrant) x 100.

Figure 4.

Human CD47 positive porcine ECs attenuate phagocytosis by baboon macrophages. (A) Representative data of FCM profile. CFSE-labeled ECs from domestic swine, MHC inbred miniature swine, MHC inbred miniature GalTKO swine, hCD46/hCD55 positive GalTKO domestic swine, and hCD47/hCD55 positive GalTKO MHC inbred miniature swine were incubated with baboon macrophages for 4 hours at 37°C. Baboon macrophages counterstained with allophycocyanin-conjugated anti-human CD14 and phagocytosis of CFSE-labeled targets were measured by FCM analysis. 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: (B left) Percentages of baboon macrophages that phagocytized pig ECs = (percentage in upper right quadrant/ percentage in upper left quadrant + percentage in upper right quadrant) x 100. (B right) Percentages of pig ECs that were phagocytized by baboon macrophages = (percentage in upper right quadrant/ percentage in upper right quadrant + percentage in lower right quadrant) x 100.

Figure 5.

Similar analysis to Fig 3 and 4 was performed using porcine podocytes. Human CD47 positive porcine podocytes attenuate phagocytosis by baboon macrophages. (A) CFSE-labeled podocytes from domestic swine MHC inbred miniature swine, hCD46/hCD55-positive GalTKO domestic swine, and hCD47/hCD55-positive GalTKO MHC inbred miniature swine were incubated with baboon macrophages for 4 h at 37°C. Representative FCM profiles are shown. (B) Phagocytic activities are shown.

Figure 6.

Masking hCD47 on hCD47Tg GalTKO ECs restores phagocytosis by baboon macrophages. FCM profiles to assess cross reactivity of anti-human CD47 ab (A) and pig CD47 ab (B) that were used in this assay using GalTKO, baboon and human PBMCs. (C) CFSE-labeled hCD47 Tg GalTKO PAECs pre incubated with isotype IgG ab or anti-human CD47 ab, then co-cultured with baboon macrophages for 4 h at 37°C. Phagocytosis was restored when human CD47+ porcine ECs was pre-incubated with anti-human CD47 ab (Fig. 6C right). (D) Anti-pig CD47 ab or isotype control IgG ab was pre-incubated with GalTKO pig ECs. Anti-pig ab did not change phagocytosis of GalTKO ECs by baboon macrophages.