Kidney transplantation from triple-knockout pigs expressing multiple human proteins in cynomolgus macaques

David Ma¹ | Takayuki Hirose¹ | Grace Lassiter¹ | Hajime Sasaki¹ | Ivy Rosales² | Taylor M. Coe¹ | Charles G. Rickert¹ | Rudy Matheson¹ | Robert B. Colvin² | Wenning Qin³ | Yinan Kan³ | Jacob V. Layer³ | Violette B. Paragas³ | Kathryn Stiede³ | Katherine C. Hall³ | Michele E. Youd³ | Luis M. Queiroz³ | William F. Westlin³ | Michael Curtis³ | Luhan Yang³ | James F. Markmann¹,³ | Tatsuo Kawai¹

¹Center for Transplantation Sciences, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts
²Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA
³eGenesis Inc., Cambridge, Massachusetts

Correspondence
Tatsuo Kawai, Center for Transplantation Sciences, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA.
Email: tkawai@mgh.harvard.edu

Funding Information
eGenesis Inc

Abstract
Porcine cells devoid of three major carbohydrate xenoantigens, αGal, Neu5GC, and SDa (TKO) exhibit markedly reduced binding of human natural antibodies. Therefore, it is anticipated that TKO pigs will be better donors for human xenotransplantation. However, previous studies on TKO pigs using old world monkeys (OWMs) have been disappointing because of higher anti-TKO pig antibodies in OWMs than humans. Here, we show that long-term survival of renal xenografts from TKO pigs that express additional human transgenes (hTGs) can be achieved in cynomolgus monkeys. Kidney xenografts from TKO-hTG pigs were transplanted into eight cynomolgus recipients without pre-screening for low anti-pig antibody titers. Two recipients of TKO-A xenografts with low expression of human complement regulatory proteins (CRPs) survived for 2 and 61 days, whereas six recipients of TKO-hTG xenografts with high CRP expression (TKO-B) survived for 15, 20, 71, 135, 265, and 316 days. Prolonged CD4⁺ T cell depletion and low anti-pig antibody titers, which were previously reported important for long-term survival of αGal knock-out (GTKO) xenografts, were not always required for long-term survival of TKO-hTG renal xenografts. This study indicates that OWMs such as cynomolgus monkeys can be used as a relevant model for clinical application of xenotransplantation using TKO pigs.

Keywords
immunosuppression/immune modulation, translational research/science, xenobody, xenograft, xenotransplantation

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2021 The Authors. American Journal of Transplantation published by Wiley Periodicals LLC on behalf of The American Society of Transplantation and the American Society of Transplant Surgeons.
1 | INTRODUCTION

The tremendous success of transplantation as a life-saving therapy for end-stage organ failure has increased organ demand. Currently, over 108,000 individuals await organs but fewer than 40,000 transplants are performed yearly in the US. To address this unmet healthcare need, the potential use of porcine organs for human transplantation has been sought. A major barrier to xenotransplantation exists in the reactivity of human natural antibodies to several carbohydrate xenoantigens expressed on porcine cells. The recent advent of CRISPR/Cas9 gene editing technology has made it possible to efficiently inactivate multiple genes that encode enzymes responsible for synthesizing these xenoantigens and to simultaneously address pig-primate coagulation and complement pathway incompatibilities by transgenically expressing various human genes. With this extremely powerful technology, “triple knock-out” (TKO) pigs devoid of three major carbohydrate xenoantigens (αGal, Neu5GC, and SDA) have recently been developed. Since the binding of human natural antibodies to TKO cells is significantly lower compared to GTKO pigs, the TKO pig is expected to be a better donor for human xenotransplantation. In the current study, we transplanted renal xenografts from TKO pigs that expressed multiple human transgenes (hTGs) in cynomolgus monkeys, demonstrating that long-term, rejection-free renal xenograft survival can be achieved with TKO-hTG pigs transplanted in nonhuman primates. Further modifications of the porcine genome, refinement of the immunosuppressive protocol, as well as effective infection prophylaxis may improve the consistency of long-term survival.

2 | MATERIALS AND METHODS

2.1 | Production of pigs with TKO and multiple human transgenes

Porcine cells carrying TKO and hTGs were generated and analyzed as reported previously and described in brief below.

2.1.1 | Gene modification

CRISPR/Cas9 was used to generate GGTA1, B4GALNT2, and CMAH gene-edited cells. Guide RNA (gRNA) sequences included GCTGCTTGT CTCACAAGTA targeting GGTA1, AGCTGGAAGACTTCCAGG targeting B4GALNT2, and GAAGCTGCCAATCTCAAGGA targeting CMAH. Human transgenes were cloned into a transposon construct and integrated into the porcine genome mediated by PiggyBAC transposase. In both constructs A and B, the human EEF1A1 promoter directed expression of the complement regulatory genes (CD46, CD55, CD59) and the CAG promoter directed expression of the other genes (HLA-E, B2M, CD47). In addition, construct A carried the PD-L1 gene linked 3’ to CD47 by a 2A sequence.

2.1.2 | Somatic cell nuclear transfer

Cumulative cells were removed 42 h after the onset of maturation. Mature oocytes were enucleated by aspirating MII plate prior to use as recipient cytoplasm. A single fibroblast was transferred into the perivitelline space of the recipient oocyte. The oocyte cytoplasm and fibroblast were fused with an electric pulse of 1.6 kV/cm for 35 μs. The electrofusion was followed by chemical activation and cell cycle synchronization. After that, the cloned embryos were moved into culture media. The surrogate gilts were synchronized by oral administration of progesterone analog Matrix (Merck Animal Health) for 17 to 19 days. Cloned embryos were cultured up to 4 days before selected for transfer into an estrus synchronized surrogate. Pregnancies were confirmed by ultrasound on day 34 following transfer. Cloned piglets were delivered at day 117 of pregnancy by c-section. Porcine endogenous retrovirus (PERV) was not removed from pigs in the current study.

2.2 | Characterization of protein expression by IHC and immunofluorescence detection

Cryosections of 8-week-old WT and transgenic porcine kidney tissues were used to characterize the protein expression of the following transgenes: CD55, CD59, and HLA-E. Briefly, cryosections were air-dried, fixed with 10% formalin, blocked, and then stained using primary antibodies followed with secondary antibodies conjugated to Alexa 647. Primary and secondary antibodies were diluted in TBS buffer plus 5% goat serum and the incubation times are 2 h and 30 min, respectively.

Formalin-fixed paraffin-embedded (FFPE) sections of 8-week-old WT and transgenic porcine kidney tissues were used to characterize the protein expression of the following transgenes: CD46, CD47, and PD-L1. Briefly, the FFPE sections were deparaffinized in xylene and re-hydrated in a graded alcohol series; 100%, 95%, and 80% for 2–3 min. Heat-induced epitope retrieval was performed using Citrate Buffer, pH 6.0 (for CD46 and CD47) and EDTA, pH 8.0 (for PD-L1) in a PT module preheated to 65°C. The cycle setting for epitope retrieval was set for 10 min at 102°C (with the no boil feature activated) and a final cool down step to 65°C. Endogenous peroxidases were inactivated using Peroxidized 1 followed with a blocking step in TBS plus 10% goat serum. Tissues were then stained with primary antibodies diluted in TBS plus 5% goat serum for 1 h. For detection, a goat anti-rabbit HRP conjugate and the fluorescent HRP substrate, Cy5-tetramide was used.

For both cryosections and FFPE sections, nuclear staining was performed using 2 μg/ml of Hoechst 33258 (MilliporeSigma) in PBS and ProLong Glass was used as mounting medium. Tissue sections were imaged using a Zeiss Axioskop z1 fluorescence motorized slide scanner using the same parameters for all tissue types that were fixed and processed similarly. Images were generated using the Zeiss Zen Blue 3.0 analysis software.

Here is a list of reagents used: rabbit anti human CD46 (ab108307, dilution 1/500, Abcam), mouse anti human CD55 (555691, dilution 1/50, BD Biosciences), mouse anti human CD59 (ab9182,
IgG and IgM was evaluated. Each sample was measured in duplicate. Incuding non-viable cells, median fluorescent intensity (MFI) level of exclude non-viable cells. The fluorescence of the stained samples with 150 30 min. After washing with staining buffer, cells were resuspended anti-rat CD31 (TLD-3A12) (BD Bioscience) diluted 1:50 at 4°C for antibody (Jackson ImmunoResearch, Inc.) each diluted 1:100, and 1:64 at 4°C for 45 min. Cells were then washed with staining buffer and incubated with anti-human IgG and anti-human IgM secondary antibody (Jackson ImmunoResearch, Inc.) each diluted 1:100, and Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (A21237 dilution 1:500, ThermoFisher), F(ab')2-Goat anti-Mouse IgG (H+L), Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (A21237 dilution 1:500, ThermoFisher), and peroxidized 1 (PX968MM, BioCare Medical).

2.3 IgG/IgM binding analysis

Serum IgG/IgM binding to porcine endothelial cells was measured by flow cytometry. Porcine endothelial cells were isolated as previously described. Endothelial cells from each donor pig were used to measure donor-specific anti-pig IgG and IgM antibodies in the serum of each recipient at multiple time points. Each endothelial cell preparation (1 x 10^5 cells per test) was incubated with 50 µl of serum diluted 1:64 at 4°C for 45 min. Cells were then washed with staining buffer and incubated with anti-human IgG and anti-human IgM secondary antibody (Jackson ImmunoResearch, Inc.) each diluted 1:100, and anti-rat CD31 (TLD-3A12) (BD Bioscience) diluted 1:50 at 4°C for 30 min. After washing with staining buffer, cells were resuspended with 150 µl of staining buffer containing 7-AAD (BD Bioscience) to exclude non-viable cells. The fluorescence of the stained samples was analyzed using FACSVerse (BD Bioscience), and FlowJo software (Tree Star). After gating the CD31-positive cell population with excluding non-viable cells, median fluorescent intensity (MFI) level of IgG and IgM was evaluated. Each sample was measured in duplicate.

2.4 Animals

Cynomolgus monkeys (Wild captive monkeys purchased from Charles River Primates) weighing 6-11 kg (estimate age 3-8 years old) were used. GTKO/CD55 pigs were purchased from the National Swine Resource and Research Center at the University of Missouri (Columbia, MO). TKO-A (EGEN-2528) and TKO-B (EGEN-2536) were provided by eGenesis (Cambridge, MA). Pigs weighing 10–25 kg were used as the kidney donor. All surgical procedures and postoperative care of animals were performed in accordance with National Institute of Health guidelines for the care and use of primates and were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

2.5 Pig to cynomolgus monkey kidney transplantation

A central venous line was inserted through internal jugular vein 3–7 days before kidney transplantation. Through the midline incision, the kidney xenograft was transplanted intra-peritoneally by anastomosing renal vein and artery to the vena cava and abdominal aorta, respectively. Uretero-vesical anastomosis was performed by the Lich-Gregoir technique without placing a ureteral stent. Bilateral native nephrectomy was performed simultaneously. Postoperatively, the transplant kidney was monitored by urine output, ultrasound, and serum creatinine measurement twice a week. The central venous line was removed by 2–4 weeks once recipient animals have stable kidney function to avoid the risk of infection.

2.6 Immunosuppressive protocol and postoperative managements

The recipients were treated with 20 mg/kg of anti-CD20 mAb on day 0 (NIH Nonhuman Primate Reagent Resource). A couple of more doses were added to keep CD20+ cell depletion for 200 days and 5 mg/kg of rabbit anti-thymocyte globulin (rATG, NIH Nonhuman Primate Reagent Resource) on days −1 and 0 as induction therapy, followed by 20 mg/kg of weekly anti-CD40 (2C10R4, mouse-rhesus chimeric) or anti-CD154 mAb (5C8, mouse-human chimeric), both from NIH Nonhuman Primate Reagent Resource and mycophenolate mofetil (MMF, Genentech) 200 mg PO daily. All TKO recipients were treated with anti-CD154 mAb. To prevent possible thrombotic complication by anti-CD154 mAb, ketorolac 1 mg/kg were also simultaneously administered. During the first 2 months, either rapamycin (Pfizer) or tacrolimus (Astellas, intramuscular injection) with solumedrol (Pfizer) were also administered (Figure 1). Since the optimal therapeutic trough level of rapamycin or tacrolimus has not been established in cynomolgus monkeys, trough levels of these drugs were adjusted higher (10–15 ng/ml) than the human dose based on our experience in renal allograft transplantation. To avoid transfusion of blood that contains anti-pig natural antibodies, subcutaneous Epogen (Amgen) injection was started after transplantation. Daily administration was initially necessary as recombinant human erythropoietin may not cross-react well in monkeys. Epogen was then tapered weekly during the first 2–3 months, after which it was administered as necessary to maintain hemoglobin >9.5 g/dl. As prophylaxis for CMV infection, ganciclovir 5 mg/kg was administered intramuscularly on days 0–90. If clinically necessary, irradiated whole blood from blood donors with identical blood type was administered. Natural antibodies were not removed from these blood products. Anticoagulation with Lovenox (d0-2) was administered in the first three recipients (A1, A2, and B1).

2.7 Lymphocyte subsets analyses

Peripheral blood mononuclear cells (PBMCs) were labeled with a combination of the following mAbs: CD3 (SP34-2), CD4 (L200), CD8 (SK1), CD21 (B-ly4), CD27 (M-T271), CD28 (CD28.2), CD95 (DX2), NKG2a, CD16, and IgM (G20-127) (BD Pharmingen), CD20 (2H7) (BioLegend) and FOXP3 (236A/E7) (eBioscience, Inc.), Bax (2D2)
2.8 | Histological analyses

Protocol renal biopsies were obtained every 2–4 months in recipients with stable function as well as whenever a rise in serum creatinine occurred. Tissue was processed for light microscopy and a portion frozen for immunofluorescence staining. Other organs obtained surgically (lymph nodes, native kidney, and spleen) were similarly processed. Following euthanasia of a monkey, a complete autopsy was performed for histopathologic examination of the renal xenograft, lymph nodes, heart, lung, liver, pancreas, thymus, and skin. Xenograft H&E and PAS-stained samples were scored by current Banff criteria including C4d deposition by immunohistochemistry.11

3 | RESULTS

3.1 | A preliminary study with GTKO/CD55 xenografts revealed superior graft survival with anti-CD154 mAb over anti-CD40 mAb

To establish the basic immunosuppressive regimen, we compared anti-CD40 or anti-CD154 monoclonal antibody (mAb)-based regimens using xenografts from GTKO pigs with expression of the human CD55 transgene (GTKO/CD55). In both groups, recipients were treated with induction therapy comprising rATG and anti-CD20 mAb, followed by weekly anti-CD40 or anti-CD154 mAb, and daily MMF. During the first 2 months, rapamycin and solumedrol were also administered (Figure 1).

Two recipients treated with anti-CD40 mAb rapidly lost their kidney xenografts due to either rejection or thrombotic microangiopathy (TMA) by day 15, while two recipients treated with anti-CD154 mAb survived longer, until they were euthanized on days 76 and 93, due to antibody-mediated rejection and weight loss without rejection (Table 1; Figure 2). Based on the observations in this preliminary study, anti-CD154 mAb was used in the subsequent transplants with TKO donors.

3.2 | Expression of human proteins in TKO-A (EGEN-2528) and TKO-B (EGEN-2536)

Human protein expression was determined by flow cytometry using ear punch–derived cells from two piglet payloads, EGEN-2528 (namely TKO-A) and EGEN-2536 (TKO-B) and the results are summarized in Table 2. Figure 3A shows the expression levels of each human protein, relative to levels on wild-type non-edited pig cells (WT). Expression of complement regulatory genes (CRPs) (CD46, CD55, and CD59) was low in TKO-A, while their expression in TKO-B cells was similar to human umbilical vein endothelial cells (HUVEC). On the other hand, expression of HLA-E/B2M and CD47 in TKO-A cells was similar to that in humans, but lower in TKO-B. PDL-1 was expressed in TKO-A but absent in TKO-B.

Similar to ear punch–derived cells, immunohistochemistry staining of TKO-B kidneys showed very high expression of hCD46 and moderate expression of hCD55, hCD59 HLA-E, and hCD47. In contrast, TKO-A kidneys expressed hCD46 and HLA-E at a low level, hCD47 moderately, and PD-L1 at a higher level but no expression of hCD55 and hCD59 (Figure 3B).
| Donor | Rapa\(^1\)/Tacro\(^2\)/CB\(^3\) | Recipient ID | Graft survival\(^4\) | Complication | Biopsy and necropsy | Banff scores |
|-------|------------------|--------------|---------------------|--------------|---------------------|---------------|
| GTKO CD55 | Rapa aCD40 | R1 | 11 | Wound dehiscence | D11 TCMR, AAMR | +++ 3 2 2 0 1 1 0 0 0 1 |
| R2 | 15 | D15 No rejection | + 0 1 1 0 1 1 0 0 0 |
| GTKO CD55 | Rapa aCD154 | R3 | 76 | Wound dehiscence | D76 AAMR, CAMR, C4d+ | 2 0 1 3 0 3 0 0 0 3 |
| R4 | 93 | D51 No rejection; C4d+ | - 0 0 0 0 0 0 0 0 3 |
| TKO-A | Rapa aCD154 | A1 | 2 | Vascular thrombosis | D2 No rejection | + 0 0 0 0 0 0 0 0 0 0 |
| None aCD154 | A2 | 61 | None | D50 AAMR\(^5\), C4d+ | - 1 0 0 0 0 0 0 0 3 |
| TKO-B | Rapa aCD154 | B1 | 15 | Gross hematuria | D15 No rejection, C4d+ | - 0 0 0 0 0 0 0 0 3 |
| Vascular thrombosis | Wound dehiscence | D2 No rejection, C4d+ | - 0 0 0 0 0 0 0 1 |
| B2 | 20 | D20 Infarction, C4d+ | - x\(^8\) x x x x x x |
| B3 | 71 | D27 No rejection | - 0 0 0 0 0 3 1 0 1 |
| Wound dehiscence | Persistent anemia | D71 No rejection | +++ 0 0 0 0 0 1 1 0 1 |
| B4 | 265 | D70 No rejection, C4d+ | - 0 1 0 0 0 1 1 0 2 |
| Subcutaneous abscess | Parvovirus infection | D126 No rejection, C4d+ | - 0 0 0 0 0 1 0 2 |
| B5 aCD154 | B5 | 135 | Recurrent UTI\(^10\) | D28 No rejection | - 0 0 0 0 0 0 0 0 0 |
| Tacro | B6 | 316 | Recurrent UTI | D47 No rejection | - 0 0 0 0 0 0 0 0 0 |
| D118 | No rejection | - 1 0 0 0 0 0 0 0 |
| D183 | No rejection | - 0 0 0 0 0 0 1 1 0 |
| D217 | No rejection | - 0 0 0 0 0 1 0 0 0 |
| D228 | Early CAMR, C4d+ | + 3 1 0 0 2 1 2 1 0 0 |
| D245 | CAMR, TCMR, C4d+ | + 2 2 1 3 2 3 1 1 2 3 |
| D265 | CAMR, TCMR, C4d+ | + 2 2 1 3 2 3 1 1 2 3 |
| D278 | Early CAMR, C4d+ | + 0 0 1 0 0 0 1 2 1 0 3 |
Immunosuppression and the transplant outcomes of TKO-A and TKO-B recipients are summarized in Table 1. All recipients were treated with the anti-CD154-based regimen. Rapamycin was used in A1 and the first four recipients of TKO-B but it was replaced with tacrolimus in B5 and B6 owing to the side effects including wound dehiscence, subcutaneous abscess formation observed in some recipients treated with rapamycin (Table 1). A2 was treated with neither rapamycin nor tacrolimus.

The first recipient (A1) had a high anti-donor IgM antibody titer (5.6 times higher than no serum control) (Figure 4A) and received a TKO-A xenograft. This monkey rapidly lost xenograft function by day 2 (Figure 4B). The autopsy showed vascular thrombosis due to endothelial injury and TMA (Table 1). A2, in which anti-donor antibody titers were lower than A1 (Figure 4A), did well until around day 50 despite receiving neither rapamycin nor tacrolimus. However, he started to develop anti-donor antibodies thereafter and eventually lost xenograft function on day 61 (Figure 4B) due to T cell–mediated rejection (TCMR) and acute antibody-mediated rejection (AAMR) (Table 1). Treatment without rapamycin or tacrolimus in this recipient may have contributed to TCMR observed on final pathology.

We then tested TKO-B which expressed higher hCRPs in the subsequent six monkeys. B1 was complicated with hydronephrosis due to gross hematuria with clots in the ureter and bladder due to excessive anticoagulation by Lovenox which was initially included to inhibit thrombogenic responses against the xenograft. Despite improved serum creatinine levels after revision of the ureter, this recipient continued to have hydronephrosis and eventually lost its xenograft function by day 15 (Figure 4B). There was no significant change in anti-donor antibody titers posttransplant (Figure 4A) and the autopsy showed focal tubular necrosis and interstitial hemorrhage without rejection or TMA (Table 1).

B2 initially did well but lost kidney function on day 20 (Figure 4B) due to acute onset of graft thrombosis. There was no significant change in anti-donor pig antibody titers and platelet counts (Figure 4A) and the cause of graft thrombosis could not be concluded due to global infarction (Table 1).

B3 initially did well with no rejection or TMA in the biopsy on day 27. Although anti-donor antibody titers remained low, this recipient started to have thrombocytopenia after day 30 (Figure 4A) and eventually lost his graft function on day 71 due to extensive TMA but without TCMR or antibody-mediated rejection (AMR) (Table 1).

On the other hand, B4 did well over 200 days with normal kidney function (Figure 4B), despite significantly high pretransplant anti-donor IgG and IgM antibodies levels (Figure 4A). A biopsy taken on day 203 showed no rejection or TMA (Table 1; Figure 5A,B). However, this recipient developed parvovirus infection with rapidly progressive anemia after day 200 (Figure 4B), requiring multiple blood transfusions and reduction of immunosuppression. This resulted in elevation of anti-donor antibody (Figure 4A) and a biopsy on day 237 showed

| TABLE 1 | (Continued) |
|---|---|
| TMA | + |
| Biopsy and necropsy | Pyelonephritis |

---

**TABLE 1**

| Banff scores | 3 | 3 |
|---|---|---|
| g | 3 | 3 |
| C4d | + | + |
| v | 1 | 1 |
| ptc | 0 | 0 |
| cg | 2 | 2 |
| ct | 1 | 1 |
| dv | 0 | 0 |
| cv | 3 | 3 |
| TMA | + | + |
| Graft survival | 4 |
| Complication | 3 3 1 3 2 3 2 1 3 2 |
| Biopsy and necropsy | 1 |
| TMA | + |
| Banff scores | 3 3 1 3 2 3 2 1 3 2 |

---

**Recipient ID**

- **A1**: Had a high anti-donor IgM antibody titer (5.6 times higher than no serum control) and received a TKO-A xenograft. This monkey rapidly lost xenograft function by day 2. The autopsy showed vascular thrombosis due to endothelial injury and TMA.
- **A2**: Initially did well but lost xenograft function on day 61 due to T cell–mediated rejection and acute antibody-mediated rejection.
- **B1**: Complicated with hydronephrosis due to gross hematuria with clots in the ureter and bladder due to excessive anticoagulation by Lovenox. Despite improved creatinine levels after revision of the ureter, hydronephrosis continued.
- **B2**: Initially did well but lost kidney function on day 20 due to acute onset of graft thrombosis.
- **B3**: Initially did well with no rejection or TMA in the biopsy on day 27. However, thrombocytopenia developed after day 30 and the biopsy showed focal tubular necrosis and interstitial hemorrhage.
- **B4**: Did well over 200 days with normal kidney function, but developed parvovirus infection requiring multiple blood transfusions and reduction of immunosuppression.

---

**Notes**

- **Rapamycin (administered up to 2 months).**
- **Tacrolimus (administered up to 2 months).**
- **Costimulatory blockade.**
- **Graft survival (days).**
- **Thrombotic microangiopathy.**
- **Acute antibody-mediated rejection.**
- **T cell–mediated rejection.**
- **Not evaluable due to necrosis.**
- **Chronic antibody-mediated rejection.**
- **Urinary tract infection.**
**TABLE 2** Molecular edits and protein expression in TKO-A and B

| Cassette                         | Gene        | Product                                | TKO-A | TKO-B |
|----------------------------------|-------------|----------------------------------------|-------|-------|
| Xenoantigen knockout             | GGTA1       | αGal                                   | KO    | KO    |
|                                  | CMAH        | Neu5Gc                                 | KO    | KO    |
|                                  | B4GALNT2    | SDa                                    | KO    | KO    |
| Complement                       | CD46        | Membrane cofactor protein              | Low   | High  |
|                                  | CD55        | Decay accelerating factor              | Low   | High  |
|                                  | CD59        | Membrane attack complex inhibitory protein | Low | High  |
| Innate and adaptive immune regulation | HLA-E/B2M | Human leukocyte antigen E               | High  | Mod   |
|                                  | CD47        | Integrin-associated protein            | High  | Mod   |
|                                  | PDL1        | Programmed death ligand-1             | High  | —     |

B6 did well over 200 days and biopsy taken on day 217 showed no rejection (Figure 5E), no TMA (Figure 5F), and no C4d deposition (Figure 5G). However, both anti-CD154 and MMF were reduced after day 250 because of recurrent UTI (Figure 4B), which was followed by rapid development of thrombocytopenia (Figure 4A). The recipient terminally lost graft function due to pyelonephritis, nephrolithiasis, and CAMR on day 316 (Figure 5H).

### 3.4 | Lymphocyte subsets and xenograft survival

To evaluate whether prolonged lymphocyte depletion is important for long-term xenograft survival, various lymphocyte subsets were compared between the short-term (<100 days, B1, B2, and B3) and long-term (>100 days, B4, B5, and B6) survivors among TKO-B recipients. Naïve (CD95<sup>-</sup>CD28<sup>+</sup>), central memory (TCM, CD95<sup>+</sup>CD28<sup>+</sup>), and effector memory (TEM, CD95<sup>+</sup>CD28<sup>-</sup>) CD4<sup>+</sup> or CD8<sup>+</sup> T cells recovered quickly by day 10, while NK cells (NKG2α<sup>−</sup>CD16<sup>−</sup>CD8<sup>−</sup>CD3<sup>−</sup>) recovered slowly by day 100–150. B cells (CD3<sup>−</sup>CD20<sup>+</sup>) were deleted from the peripheral blood up to 260 days by 2–3 doses of rituximab. There was no significant difference in these lymphocyte counts between short-term and long-term survivors but effector memory T cells were more depressed in the short-term survivors (Figure 6).

### 4 | DISCUSSION

To establish our immunosuppressive regimen, we first evaluated the anti-CD40 mAb and anti-CD154 mAb-based regimens using xenografts from GTKO/CD55 pigs. Although the number of animals tested in this preliminary study was limited, the results clearly revealed the superiority of anti-CD154 mAb over anti-CD40 mAb in the suppression of kidney xenograft rejection. This difference may be attributed to the dose of anti-CD40 mAb, 20 mg/kg/week, used in this study which was chosen as a clinically feasible dose based on our previous nonhuman primate studies in allotransplantation. However, this represents less than half the dose used by other groups in xenotransplantation and a higher dose may be necessary to...
FIGURE 3  (A) Expression of human proteins in TKO-A (EGEN-2528) and TKO-B (EGEN-2536) pig donors. Human protein expression was determined by flow cytometry using ear punch–derived cells. Histograms representing the expression of indicated transgenes. Expression of complement regulatory genes (CRPs) was low in TKO-A, while their expression was high in TKO-B cells. On the other hand, expression of HLA-E/B2M and CD47 was high in TKO-A, but lower in TKO-B. PDL-1 was expressed only in TKO-A. HUVEC, human umbilical vein endothelial cells; WT, ear punch–derived cells from wild type pigs; HLAE, HLA-E; B2M, beta-2-microglobulin. (B) Expression of human proteins in kidneys from TKO-A and TKO-B. Immunofluorescence staining of the human transgenic proteins in TKO-A (EGEN-2528), TKO-B (EGEN-2536), and wild-type kidney cryosections and FFPE sections, as described in Section 2. Nuclear counterstaining was performed with the Hoechst dye. Scale bars, 50 μm. TKO-B kidneys showed very high expression of hCD46 and moderate expression of hCD55, hCD59 HLA-E, and hCD47. In contrast, expression of CRPs in TKO-A kidneys was weak (CD46) or absent (CD55 and CD59), while expression of CD47 and PD-L1 was high.
achieve equivalent immunosuppressive effects to those induced by anti-CD154 mAb (20 mg/kg/week). One of the monkeys survived until day 93 was euthanized due to body weight loss without rejection. Although this recipient could have survived longer, we decided not to repeat this transplant in more recipients as convincing results of GTKO/CD55 xenografts treated with anti-CD154 mAb have already been reported by the Emory group.\textsuperscript{14} Based on these findings and our preliminary study, we selected an anti-CD154 mAb-based regimen to test kidney xenografts from TKO-hTG pigs.

Long-term kidney xenograft survival from GTKO/CD55 pigs has previously been reported by two groups. The group from Emory University achieved long-term renal xenograft survival only in rhesus monkeys pre-screened for low anti-pig antibody titers.\textsuperscript{14,15} They also reported that prolonged selective depletion of CD4\textsuperscript{+}T cells by anti-CD4 mAb is critically important for long-term survival of xenografts. While mechanistically interesting, anti-CD4 mAb is not clinically available, constraining clinical application of their approach. Using GTKO with CD46/CD55/CD47/EPCR/TFPI transgenes, the group from Pittsburgh University reported long-term kidney xenograft survival up to 260 days in two baboon recipients with low anti-pig antibodies.\textsuperscript{16}

Prior work using TKO pig kidneys in baboons were discouraging. The Alabama group evaluated three xenografts from TKO pigs using an anti-CD40 mAb-based immunosuppressive regimen. Multiple hTGs were also combined with TKO in their study. However, all three baboons rejected the xenografts by AMR, with two succumbing very rapidly by day 4. In vitro analyses showed that the binding of anti-TKO IgM antibodies was higher in old world monkeys (OWMs) than those in humans, although IgG anti-TKO antibody levels were similar to humans. Although the number of animals was limited, the authors concluded that the OWM is not an optimal model for evaluating xenografts from TKO pigs.\textsuperscript{5}

OWMs, like pigs, have the CMAH gene and thus express the Neu5Gc antigen and the CMAH knock-out modification may actually unveil as-yet-unidentified antigens recognized by antibodies in the blood of OWMs (but not in humans).\textsuperscript{5} Therefore, double knock-out (DKO) of GGTA1 and B4GALNT2 may be a more appropriate pig genotype for xenotransplantation using OWMs than those in humans, although IgG anti-TKO antibody levels were similar to humans. Although the number of animals was limited, the authors concluded that the OWM is not an optimal model for evaluating xenografts from TKO pigs.\textsuperscript{5}

In our study, similar to studies by others,\textsuperscript{5,18} anti-TKO pig antibodies were very high in some monkeys (Figure 3A). However, unlike the Alabama group, we report that long-term TKO kidney xenograft survival appeared possible in cynomolgus monkeys. Among TKO-B recipients, B3 is the only recipient who lost the xenograft due to TMA while on a full immunosuppressive regimen. Other TKO-B recipients, including B4 and B5 with markedly high IgG and IgM antibody, developed TMA or CAMR only after reduction of immunosuppression which was necessitated due to infectious complications. While hTGs might have played an important role to overcome high antibodies against the TKO xenografts, the significance of the hTGs remains to be defined by performing controls without hTGs. Positive C4d deposition detectable early after transplant in some TKO-B recipients may not indicate the failure of hTGs, since the human complement transgenes (CD46, CD55, and CD59) only attenuate the complement cascade after C4 activation.\textsuperscript{20,21} Meanwhile, it seems likely that xenotransplantation using TKO-hTG xenografts may be associated with better outcomes in human recipients than can be achieved in OWMs, since anti-TKO antibodies are consistently lower in humans than in OWMs.

Another difference in our TKO-hTG xenograft experiments relative to the Emory studies with GTKO was that prolonged depletion of CD4\textsuperscript{+}T cells was not required for long-term kidney xenograft survival. There was no significant difference in CD4\textsuperscript{+} T cell depletion between short-term (<100 days) and long-term survivors (>100 days) in TKO-B recipients. Despite rapid recovery of all T cell subpopulations after rATG, three recipients achieved long-term xenograft survival. One of the possible reasons why prolonged CD4\textsuperscript{+} T cell depletion was not required for long-term survival of our TKO-hTG xenografts may be inclusion of rapamycin or tacrolimus for 2 months in our immunosuppressive protocol, which was not included in the Emory protocol. Weekly administration of anti-CD154 mAb in our recipients vs. biweekly (once every 2 weeks) administration in the Emory study may also be helpful to suppress CD4\textsuperscript{+} T cells function even after the recovery of these T cells. However, interesting observation in our study has been that rapamycin or tacrolimus was not required after 2 months.

In the current study, infectious complications in the NHPs terminated their graft function. The frequent catheterization, which was necessary for collection of clean urine needed for accurate evaluation of protein content, may be a contributing factor to the multiple UTI episodes. When serious infection was observed in these monkeys, rapamycin or tacrolimus was already discontinued and these are not considered to be responsible for infection. However, prolonged B cell deletion by rituximab, weekly anti-CD154 mAb administration may have been responsible for infectious complications and optimal maintenance immunosuppressive regimen remains to be defined. In response, our posttransplant care protocol has been revised to avoid frequent ureteral catheterization and reduce the dose of anti-CD154 mAb after day 200. UTI has not been observed in the current series of xenotransplant recipients without adding any infection prophylaxis.

In conclusion, long-term survival of kidney xenografts from TKO-hTG pigs with additional rationally targeted genetic modifications was achieved in cynomolgus monkeys. Since anti-CD154 mAb is not clinically available, we are currently testing an Fc-modified anti-CD154 mAb in NHPs. Precise assessment of additional human transgenes on TKO pigs, additional deletion of PERV,\textsuperscript{22} as well as the effort to make anti-CD154 mAb clinically available, may bring us closer for potential clinical trial of xenotransplantation.\textsuperscript{5}
FIGURE 4 (A) Anti-donor IgG and IgM antibodies and platelet counts pre- and posttransplant. Antibody bindings to donor pig endothelial cells were measured by flow cytometry as described in the method. The each column shows the ratio of mean fluorescent intensity (MFI) of IgG (black) and IgM (gray) antibody binding to the background MFI without serum. Dotted lines indicate platelet counts (×10³/mm³). Antibody titers against donor endothelial cells started to elevate after reduction of immunosuppression. Severe TMA was associated with thrombocytopenia in B3 and B6. (B) Clinical courses of TKO-A and TKO-B recipients. Clinical courses and immunosuppressive medications of eight TKO-A and B recipients were depicted. Red lines indicated serum creatinine levels (mg/dl). Recipients were treated with weekly anti-CD154 mAb (green), daily MMF (yellow), daily rapamycin (pink), or tacrolimus (blue) for two months and methylprednisolone (pred, gray) for 1 month. Parvovirus infection (♦) and several episodes of bacteremia (▼) and UTI (U) were observed during the post-op course of long-term survivors (B4, B5, and B6). These infectious complications necessitated to reduce the dose of immunosuppressive medications, which resulted in terminal rejections. Bx, kidney xenograft biopsy

FIGURE 5 Histopathological findings in two long-term survivors. (A–D) (B4): Biopsy on day 203 showed no rejection (H&E, ×10) (A) and no TMA (Pas, 20×) (B). However, after reduction of immunosuppression, biopsy on day 237 showed early TCMR and focal glomerular TMA (C). Terminal rejection on d265 (D). (E–H) (B6): Biopsy taken on day 217 showed no rejection (E), no TMA (F), and no C4d deposition (G). Autopsy on day 316 showed pyelonephritis, TCMR, and CAMR (H)

FIGURE 6 Lymphocyte subsets and transplant outcome (TKO-B recipients). Absolute counts (mean ± SE) of various lymphocyte subsets in the long-term (>100 days) survivors (B4–B6, magenta) were compared with those in the short-term (<100 days) survivors (B1–B3, blue). Naïve (CD95−CD28+), central memory (TCM, CD95+CD28+), and effector memory (TEM, CD95−CD28−) T cells (CD3+CD4+ or CD3+CD8+) recovered quickly by day 10, while NK cells (NKG2a+CD16+CD8−CD3−) recovered slowly by day 100. B cells (CD3−CD20+) were deleted from the peripheral blood up to 260 days by 2–3 doses of rituximab. There was no statistically significant difference in these lymphocyte counts between short-term and long-term survivors but effector memory T cells were more depressed in the short-term survivors [Color figure can be viewed at wileyonlinelibrary.com]
ACKNOWLEDGMENTS
We acknowledge Drs. Joanne Morris and Michael Duggan for veterinary supervision and Drs. Richard Pierson and Joren Madsen for critical reading and comments. We also thank Ann Adams for editorial analysis and comment.

DISCLOSURE
The authors of this manuscript have conflicts of interest to disclose as described by the American Journal of Transplantation. Research funding was provided by eGenesis Inc. eGenesis has filed patent applications on the transgenic pig technology described in this paper. Wenning Qin, Yinan Kan, Jason V. Layer, Violette B. Paragas, Kathryn Stiede, Katherine C. Hall, Michele E. Youd, Luis M. Queiroz, William F. Westlin, Michael Curtis are employees of eGenesis Bio. and Luhan Yang is a former employee of eGenesis with current affiliation to Qihan Bio. James F. Markmann and Robert B. Colvin are consultants of eGenesis. Other authors have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
Takayuki Hirose https://orcid.org/0000-0001-7950-0961
Grace Lassiter https://orcid.org/0000-0003-3891-8581
Ivy Rosales https://orcid.org/0000-0003-0621-3202
Taylor M. Coe https://orcid.org/0000-0003-3514-6993
Charles G. Rickert https://orcid.org/0000-0003-0994-3999
Robert B. Colvin https://orcid.org/0000-0002-4493-4150
Wenning Qin https://orcid.org/0000-0001-9168-5102
Kathryn Stiede https://orcid.org/0000-0002-6065-0581
Katherine C. Hall https://orcid.org/0000-0003-1091-6074
Luhan Yang https://orcid.org/0000-0002-5501-1423
James F. Markmann https://orcid.org/0000-0002-2762-6535
Tatsuo Kawai https://orcid.org/0000-0002-6900-4603

REFERENCES
1. UNOS Transplant trends. 2020. https://unos.org/data/transplant -trends/. Accessed February 20, 2021.
2. Cooper DKC, Gaston R, Eckhoff D, et al. Xenotransplantation—the current status and prospects. Br Med Bull. 2018;125(1):5-14.
3. Nunes dos Santos RM, Carneiro D'Albuquerque LA, Reyes LM, et al. CRISPR/Cas and recombinase-based human-to-pig orthotopic gene exchange for xenotransplantation. J Surg Res. 2018;229:28-40.
4. Estrada JL, Martens G, Li P, et al. Evaluation of human and non-human primate antibody binding to pig cells lacking GGTA1/CMAH/ beta4GalNT2 genes. Xenotransplantation. 2015;22(3):194-202.
5. Yamamoto T, Iwase H, Patel D, et al. Old World Monkeys are less than ideal transplantation models for testing pig organs lacking three carbohydrate antigens (Triple-Knockout). Sci Rep. 2020;10(1):9771.
6. Yue Y, Xu W, Kan Y, et al. Extensive germline genome engineering in pigs. Nat Biomed Eng. 2021;5(2):134-143.
7. Azimzadeh AM, Byrne GW, Ezzeleab M, et al. Development of a consensus protocol to quantify primate anti-non-Gal xenoreactive antibodies using pig aortic endothelial cells. Xenotransplantation. 2014;21(6):555-566.
8. Koyama I, Kawai T, Andrews D, et al. Thrombophilia associated with anti-CD154 monoclonal antibody treatment and its prophylaxis in nonhuman primates. Transplantation. 2004;77(3):460-462.
9. Yamada Y, Boskovic S, Aoyama A, et al. Overcoming memory T-cell responses for induction of delayed tolerance in nonhuman primates. Am J Transplant. 2012;12(2):330-340.
10. Loupy A, Haas M, Roufosse C, et al. The Banff 2019 Kidney Meeting Report (I): updates on and clarification of criteria for T-cell- and antibody-mediated rejection. Am J Transplant. 2020;20(9):2318-2331.
11. Adam BA, Smith RN, Rosales IA, et al. Chronic antibody-mediated rejection in nonhuman primate renal allografts: validation of human histological and molecular phenotypes. Am J Transplant. 2017;17(11):2841-2850.
12. Oura T, Hotta K, Lei J, et al. Immunosuppression with CD40 co-stimulatory blockade plus rapamycin for simultaneous islet and kidney transplantation in nonhuman primates. Am J Transplant. 2017;17(3):646–656.
13. Mohiuddin MM, Singh AK, Corcoran PC, et al. Chimeric 2C10R4 anti-CD40 antibody therapy is critical for long-term survival of GTKO/hCD46.hTBM pig-to-primate cardiac xenograft. Nat Commun. 2016;7(1):11138.
14. Kim SC, Mathews DV, Breeden CP, et al. Long-term survival of pig-to-rhesus macaque renal xenografts is dependent on CD4 T cell depletion. Am J Transplant. 2019;19(8):2174-2185.
15. Higginbotham L, Mathews D, Breeden CA, et al. Pre-transplant antibody screening and anti-CD154 costimulation blockade promote long-term xenograft survival in a pig-to-primate kidney transplant model. Xenotransplantation. 2015;22(3):221-230.
16. Iwase H, Hara H, Ezzeleab M, et al. Immunological and physiological observations in baboons with life-supporting genetically engineered pig kidney grafts. Xenotransplantation. 2017;24(2).
17. Cui Y, Yamamoto R, Raza SS, et al. Evidence for GTKO/beta4GalNT2KO pigs as the preferred organ-source for old world nonhuman primates as a preclinical model of xenotransplantation. Transplant Direct. 2020;6(8):e590.
18. Adams AB, Kim SC, Martens GR, et al. Xenotransplantation: Relevance to studies of xenotransplantation. Xenotransplantation. 2019;26(4):e12498.
19. Zhou H, Hara H, Cooper DKC. The complex functioning of the complement system in xenotransplantation. Xenotransplantation. 2019;26(4):e12517.
20. Platt JL. C4d and the fate of organ allografts. J Am Soc Nephrol. 2002;13(9):2417-2419.
21. Niu D, Wei H-J, Lin L, et al. Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. Science. 2017;357(6357):1303-1307.

How to cite this article: Ma D, Hirose T, Lassiter G, et al. Kidney transplantation from triple-knockout pigs expressing multiple human proteins in cynomolgus macaques. Am J Transplant. 2022;22;46–57. https://doi.org/10.1111/ajt.16780