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ORIGINAL ARTICLE

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

David Ma[1] | Takayuki Hirose[1] | Grace Lassiter[1] | Hajime Sasaki[1] | Ivy Rosales[2] | Taylor M. Coe[1] | Charles G. Rickert[1] | Rudy Matheson[1] | Robert B. Colvin[2] | Wenning Qin[3] | Yinan Kan[3] | Jacob V. Layer[3] | Violette B. Paragas[3] | Kathryn Stiede[3] | Katherine C. Hall[3] | Michele E. Youd[3] | Luis M. Queiroz[3] | William F. Westlin[3] | Michael Curtis[3] | Luhan Yang[3] | James F. Markmann[1,3] | Tatsuo Kawai[1]

[1]Center for Transplantation Sciences, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts

[2]Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA

[3]eGenesis Inc., Cambridge, Massachusetts

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

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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-hTG xenografts with low expression of human complement regulatory proteins (CRPs) (TKO-A) 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, xenoantibody, xenoantigen, xenotransplantation

Abbreviations: AAMR, acute antibody-mediated rejection; AMR, antibody-mediated rejection; C4, complement component 4; CAMR, chronic antibody-mediated rejection; *CMAH*, cytidine monophospho-N-acetylneuraminic acid hydroxylase; *GGTA1*, glycoprotein α(1,3)galactosyl transferase; gRNA, guide RNA; GTKO, α(1,3)galactosyl transferase gene knock-out; hTGs, human transgenes; MMF, mycophenolate mofetil; NHP, nonhuman primates; OWM, old world monkeys; PBMC, peripheral blood mononuclear cells; TCMR, T cell–mediated rejection; TKO, triple knock-out; TMA, thrombotic microangiopathy; *β4GALNT2*, β1,4-N-acetyl-galactosaminyl transferase 2.

David Ma and Takayuki Hirose contributed equally to the study

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.[1] To address this unmet healthcare need, the potential use of porcine organs for human transplantation has been sought.[2] 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 the 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.[3] 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 *GGTA1* single knock-out (GTKO) or *GGTA1/ β4GALNT2* double knock-out (DKO) porcine cells,[4,5] 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[6] 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 CTCAACTGTAA targeting *GGTA1*, AGCTCGAACACTTTCAGAGG 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

Cumulus 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 rehydrated 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 Peroxidazed 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-tyramide 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 Axioscan z.1 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,

dilution 1/50, Abcam), mouse anti HLA-E (LS-C179648, dilution 1/250, LSBio), rabbit anti human CD47 (ab226837 dilution 1/100, Abcam), rabbit anti human PD-L1 (13684 dilution 1/200, Cell Signaling Technology), EnVision + System-HRP Labelled Polymer Anti-Rabbit (K400311-2, Dako), F(ab')2-goat anti rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 647 (A48285 dilution 1/1000, ThermoFisher), F(ab')2-goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (A21246 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 peroxidazed 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.[7] 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 (1×10^5 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.[8] 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.[9] 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)

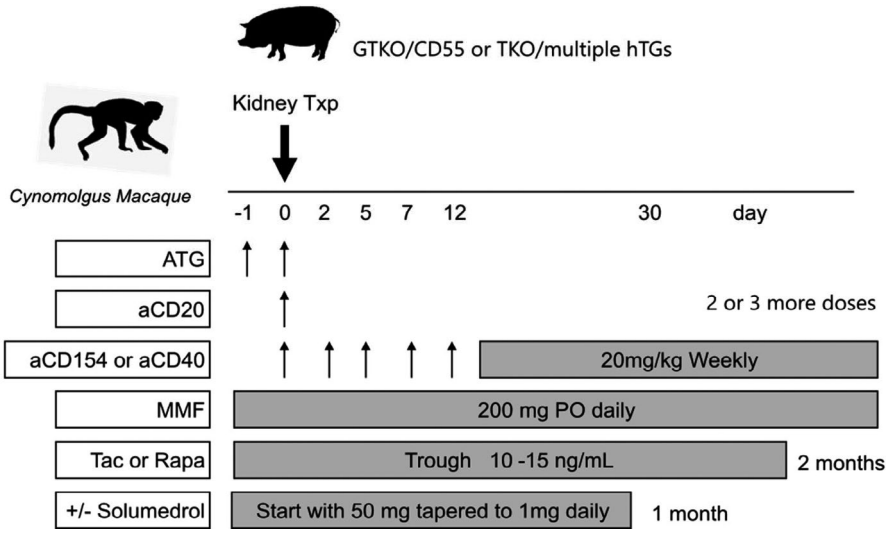


FIGURE 1 Immunosuppressive regimen. After induction with ATG and anti-CD20 (aCD20) mAb, recipients received renal xenograft from either GTKO/CD55 or TKO/hTG pigs. After transplantation, either anti-CD40 (aCD40) or anti-CD154 (aCD154) mAb on days 0, 2, 5, 7,12 and weekly (20 mg/kg), daily mycophenolate mofetil (MMF) and a 2-month course of daily tacrolimus (Tac) or rapamycin (Rapa) were administered. Methylprednisolone was also administered intramuscularly for 1 month. One to two additional doses of anti-CD20 mAb were administered to maintain $CD20^+$ B cell depletion at least until day 100

(BioLegend). The fluorescence of the stained samples was analyzed using FACS Verse (BD Biosciences) and Accuri flow cytometers (BD Pharmingen), and FlowJo software (Tree Star).

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[10] 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, respectively (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).

TABLE 1 Summary of transplant outcomes

Donor	Rapa[1]/ Tacro[2] CB[3]	Recipient ID	Graft survival[4]	Complication	Biopsy and necropsy		TMA[5]	Banff scores									
								g	i	t	v	ptc	cg	ci	ct	cv	C4d
GTKO CD55	Rapa aCD40	R1	11		D11	TCMR, AAMR	+++	3	2	2	0	1	1	0	0	0	1
		R2	15		D15	No rejection	+	0	1	1	0	1	1	1	1	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	0	3
					D93	No rejection; C4d+	−	0	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[6], C4d+	−	1	0	0	0	0	0	0	0	0	3
					D61	TCMR[7], AAMR, C4d+	+	2	2	1	1	2	0	0	0	0	3
TKO-B	Rapa aCD154	B1	15	Gross hematuria Hydronephrosis Wound dehiscence	D15	No rejection, C4d+	−	0	0	0	0	0	0	0	0	0	3
		B2	20	Vascular thrombosis	D2	No rejection, C4d+	−	0	0	0	0	0	0	0	0	0	1
					D20	Infarction, C4d+	−	x[8]	x	x	x	x	x	x	x	x	
		B3	71	Wound dehiscence Persistent anemia	D27	No rejection	−	0	0	0	0	0	0	3	1	0	1
					D71	No rejection	+++	0	0	0	0	0	0	1	1	0	1
		B4	265	Subcutaneous abscess Parvovirus infection	D70	No rejection, C4d+	−	0	1	0	0	0	0	1	1	0	2
					D126	No rejection, C4d+	−	0	0	0	0	0	0	1	0	0	2
					D203	Early CAMR[9], C4d+	−	1	0	0	0	0	1	1	1	0	1
					D237	Early CAMR, C4d+	+	0	0	0	0	0	1	1	1	0	3
					D265	CAMR, TCMR, C4d+	+	2	2	1	3	2	3	1	1	2	3
	Tacro aCD154	B5	135	Recurrent UTI[10]	D28	No rejection	−	0	0	0	0	0	0	0	0	0	0
					D108	Early CAMR, C4d+	−	3	1	0	0	2	1	2	1	0	1
					D135	Early CAMR Pyelonephritis	+	3	1	0	0	2	1	2	1	0	0
		B6	316	Recurrent UTI	D47	No rejection	−	0	0	0	0	0	0	0	0	0	0
					D118	No rejection	−	1	0	0	0	0	0	0	0	0	0
					D183	No rejection	−	0	0	0	0	0	0	1	1	0	0
					D217	No rejection	−	0	0	1	0	0	0	2	1	0	0
					D278	Early CAMR, C4d+	+	0	0	1	0	0	1	2	1	0	3

(Continues)

TABLE 1 (Continued)

Donor	Rapa[1]/ Tacro[2] CB[3]	Recipient ID	Graft survival[4]	Complication	Biopsy and necropsy	TMA[5]	Banff scores								
							g	i	t	v	ptc	cg	ci	ct	cv	C4d
					D310 CAMR, C4d+	+	3	0	1	0	2	3	2	1	0	3
					D316 CAMR,TCMR, C4d+ Pyelonephritis	+	3	3	1	3	2	3	2	1	3	2

[1]Rapamycin (administered up to 2 months).
[2]Tacrolimus (administered up to 2 months).
[3]Costimulatory blockade.
[4]Graft survival (days).
[5]Thrombotic microangiopathy.
[6]Acute antibody-mediated rejection.
[7]T cell–mediated rejection.
[8]Not evaluable due to necrosis.
[9]Chronic antibody-mediated rejection.
[10]Urinary tract infection.

3.3 | Xenograft transplant outcomes in recipients of TKO-A and TKO-B

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

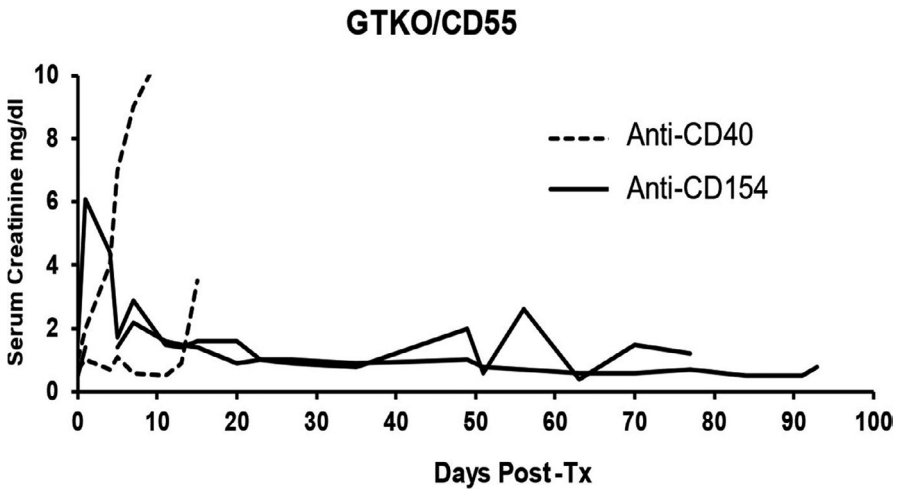


FIGURE 2 Serum creatinine in recipients transplanted with GTKO/CD55 xenografts. Two recipients treated with the anti-CD154 mAb-based regimen survived for 76 and 93 days, while both recipients treated with anti-CD40 mAb quickly lost their xenografts due to either rejection or TMA on day 11 and 15

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	—

early CAMR (cg1) and focal glomerular TMA (Figure 5C). The graft was terminally rejected on day 265 with chronic antibody-mediated rejection (CAMR) and TCMR (Table 1; Figure 5D).

B5 initially did well, despite very high anti-pig donor IgG and IgM antibody titers (Figure 4A) with a biopsy on day 28 showing no rejection or TMA (Table 2). However, his immunosuppression was progressively reduced owing to repeated bacteremia and urinary tract infection (UTI) (Figure 4B), after which anti-donor IgG and IgM antibodies started to rise (Figure 4A). A biopsy on day 108 showed early CAMR (Table 1) and the kidney graft function was eventually lost on day 135 (Figure 4B), stemming from pyelonephritis and CAMR with TMA.

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 ($CD95^-CD28^+$), central memory (TCM, $CD95^+CD28^+$), and effector memory (TEM, $CD95^+CD28^-$) $CD4^+$ or $CD8^+$ T cells recovered quickly by day 10, while NK cells ($NKG2a^+CD16^+CD8^+CD3^-$) recovered slowly by day 100–150. B cells ($CD3^-CD20^+$) 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.[12] However, this represents less than half the dose used by other groups in xenotransplantation[5,13] and a higher dose may be necessary to

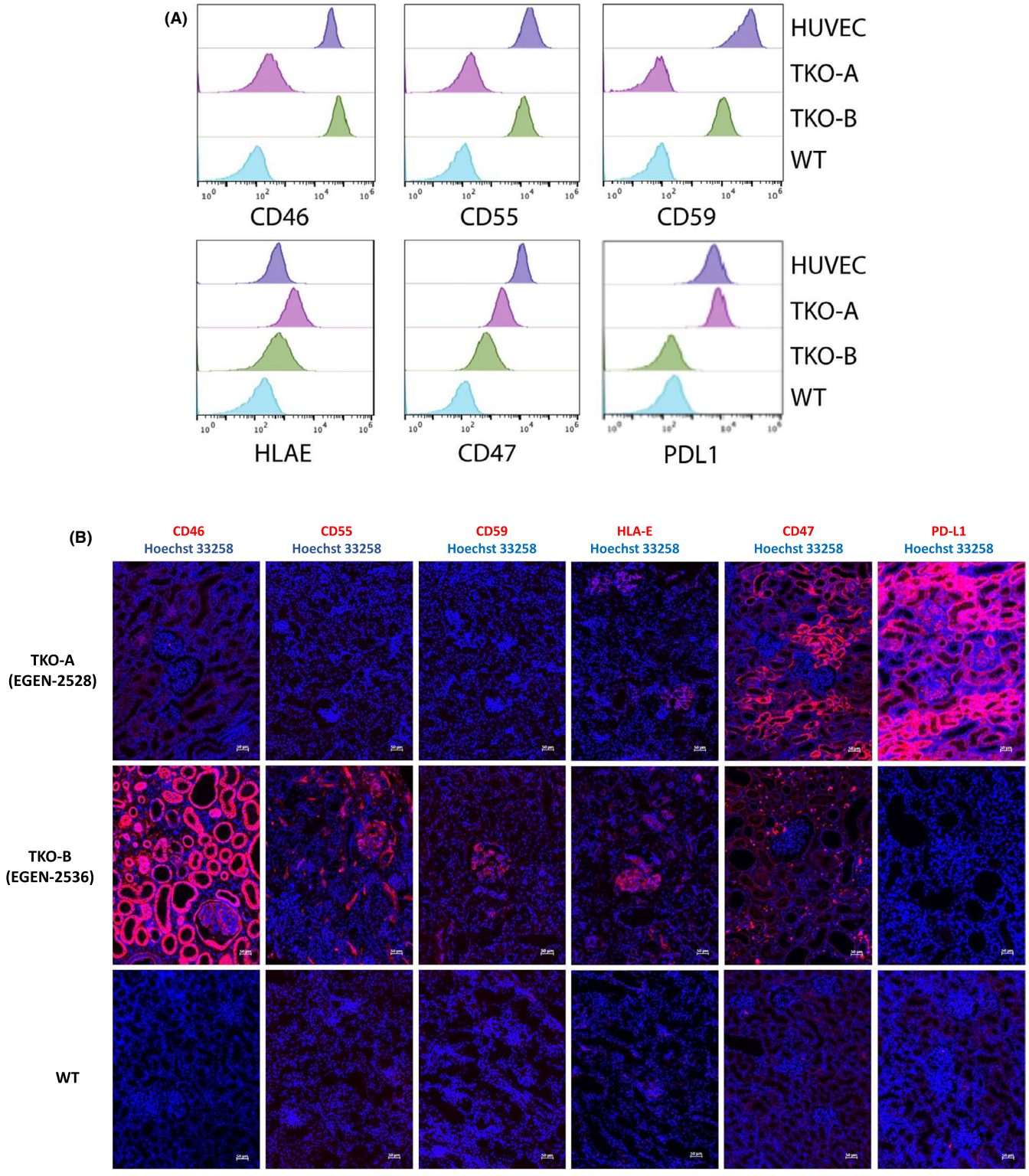


FIGURE 3 (A) Expression of human proteins in TKO-A (EGEN-2528) and TKO-B (EGEN-2536) pig donors. Human protein expression was determined by flow cytometry using ear punch–derived cells. Histograms representing the expression of indicated transgenes. Expression of complement regulatory genes (CRPs) was 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.[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.[14,15] They also reported that prolonged selective depletion of $CD4^+$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.[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.[5]

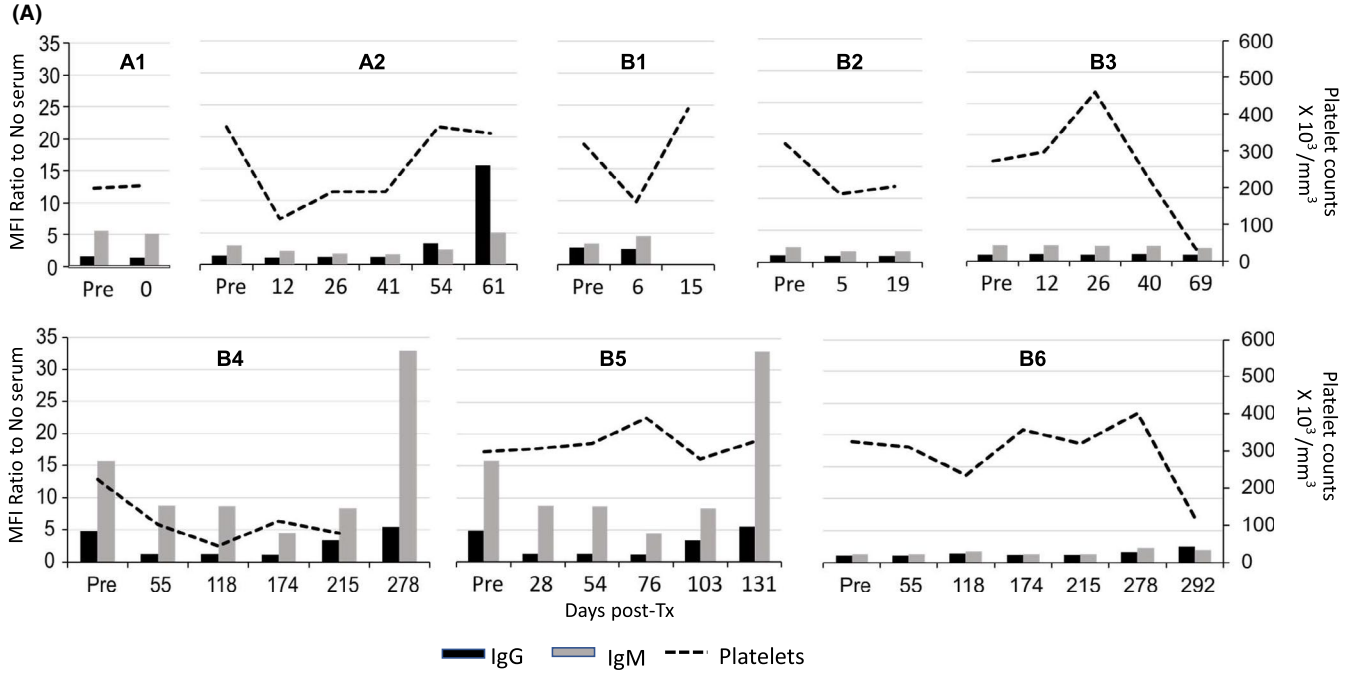
OWMs, like pigs, have the *CMAH* gene and thus express the Neu5GC antigen and the *CMAH* knock-out modification may actually unveil as-yet-unidentified antigens recognized by antibodies in the blood of OWMs (but not in humans).[5] Therefore, double knock-out (DKO) of *GGTA1* and B4GALNT2 may be a more appropriate pig genotype for xenotransplantation using OWMs.[17] To test this hypothesis, the Emory group recently evaluated kidney xenografts from DKO pigs and reported long-term survival (100 and 435 days) in two recipients. However, long-term survival was not consistently observed even with DKO and three succumbed to rapid rejection (<1 week) and another recipient only survived up to 35 days.[18] Because of the complexity and difference in expression of carbohydrate antigens between humans and NHPs,[19] there is no ideal preclinical NHP model to test TKO pigs. Nevertheless, since TKO pigs are the best available genetically modified donors for humans, evaluating TKO xenografts using NHPs remains important for possible clinical application.

In our study, similar to studies by others,[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.[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 $CD4^+$T cells was not required for long-term kidney xenograft survival. There was no significant difference in $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ 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,[22] as well as the effort to make anti-CD154 mAb clinically available, may bring us closer for potential clinical trial of xenotransplantation.[6]


(A)
A1
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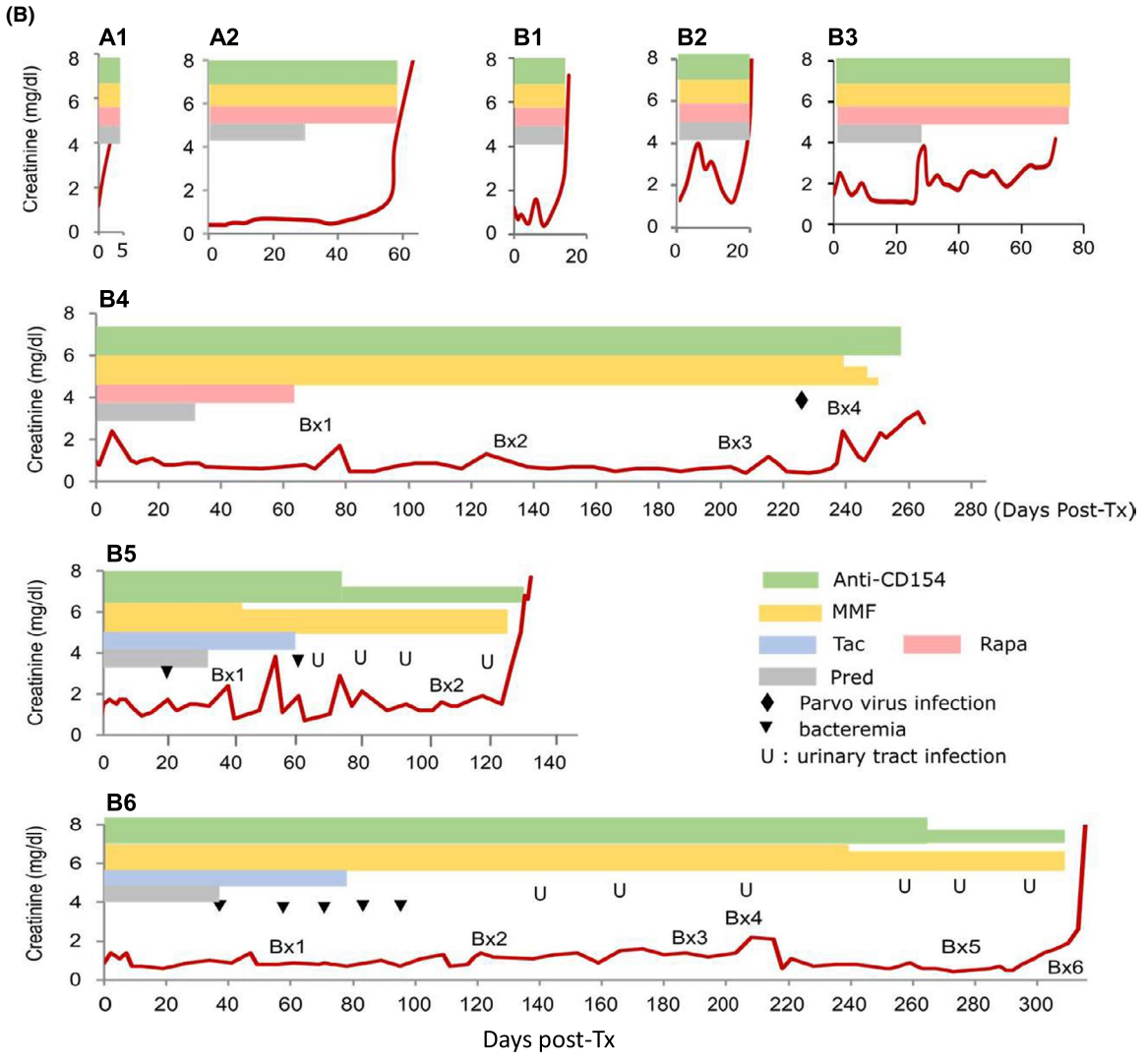
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Bx6
U
(Days Post-Tx)
Anti-CD154
MMF
Tac
Rapa
Pred
Parvo virus infection
bacteremia
U : urinary tract infection
Days post-Tx

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 ($\times 10^3/mm^3$). 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

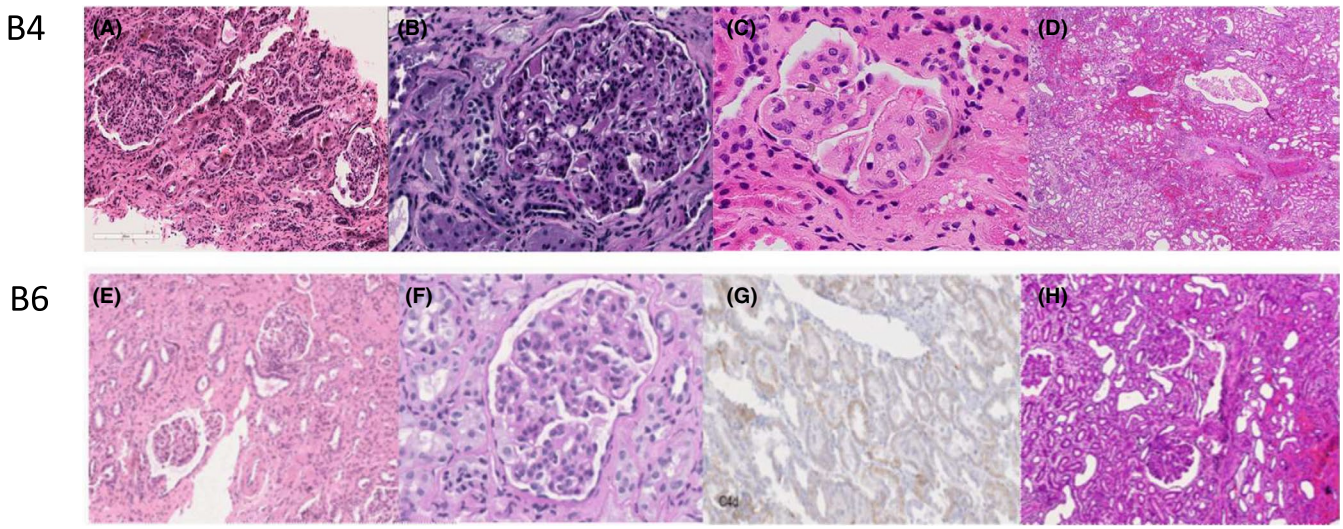

FIGURE 5 Histopathological findings in two long-term survivors. (A–D) (B4): Biopsy on day 203 showed no rejection (H&E, ×10) (A) and no TMA (Pas, 20×) (B). However, after reduction of immunosuppression, biopsy on day 237 showed early TCMR and focal glomerular TMA (C). Terminal rejection on d265 (D).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

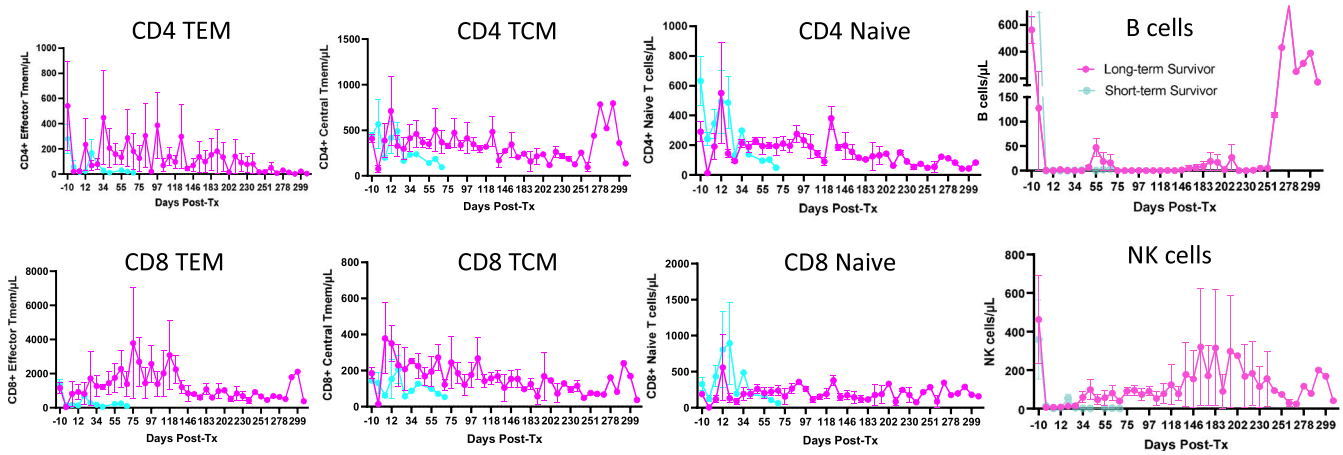

FIGURE 6 Lymphocyte subsets and transplant outcome (TKO-B recipients). Absolute counts (mean ± SE) of various lymphocyte subsets in the long-term (>100 days) survivors (B4–B6, magenta) were compared with those in the short-term (<100 days) survivors (B1–B3, blue). Naïve ($CD95^-CD28^+$), central memory (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]

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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, Jacob 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/0000-0002-0605-0581
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

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