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*Xenotransplantation*. 2015 ; 22(3): 221–230. doi:10.1111/xen.12166.**Pre-transplant antibody screening and anti-CD154 costimulation blockade promote long-term xenograft survival in a pig-to-primate kidney transplant model****Laura Higginbotham<sup>1</sup>, Dave Mathews<sup>1</sup>, Cynthia A. Breeden<sup>1</sup>, Mingqing Song<sup>1</sup>, Alton Brad Farris III<sup>2</sup>, Christian P. Larsen<sup>1</sup>, Mandy L. Ford<sup>1</sup>, Andrew J. Lutz<sup>3</sup>, Matthew Tector<sup>4</sup>, Kenneth A. Newell<sup>1</sup>, A. Joseph Tector<sup>3</sup>, and Andrew B. Adams<sup>1</sup>**<sup>1</sup>Department of Surgery, Emory Transplant Center, Emory University School of Medicine, Atlanta, GA<sup>2</sup>Anatomic Pathology, Emory University School of Medicine, Atlanta, GA<sup>3</sup>Department of Surgery, Indiana University Health Transplant Institute, Indiana University School of Medicine, Indianapolis, IN<sup>4</sup>Indiana University Health Transplant Department, Indianapolis, IN, USA**Abstract**

Xenotransplantation has the potential to alleviate the organ shortage that prevents many patients with end-stage renal disease from enjoying the benefits of kidney transplantation. Despite significant advances in other models, pig-to-primate kidney xenotransplantation has met limited success. Preformed anti-pig antibodies are an important component of the xenogeneic immune response. To address this, we screened a cohort of 34 rhesus macaques for anti-pig antibody levels. We then selected animals with both low and high titers of anti-pig antibodies to proceed with kidney transplant from galactose- $\alpha$ 1,3-galactose knockout/CD55 transgenic pig donors. All animals received T-cell depletion followed by maintenance therapy with costimulation blockade (either anti-CD154 mAb or belatacept), mycophenolate mofetil, and steroid. The animal with the high titer of anti-pig antibody rejected the kidney xenograft within the first week. Low-titer animals treated with anti-CD154 antibody, but not belatacept exhibited prolonged kidney xenograft survival (>133 and >126 vs. 14 and 21 days, respectively). Long-term surviving animals treated with the anti-CD154-based regimen continue to have normal kidney function and preserved renal architecture without evidence of rejection on biopsies sampled at day 100. This description

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**Author contributions**

The manuscript has been revised and approved by all authors. LH, DM, and ABA performed the in vivo transplant studies. LH and ABA drafted the article. ABF, MS, and AJL performed the histologic studies and analysis. LH, DM, and CB performed the flow cytometry. ABA, MT, MLF, KAN, and AJT participated in the concept and design of the experiments.

**Disclosures**

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:  
Figure S1. Pre-transplant, anti-GGTA1KO IgG and IgM titers.

of the longest reported survival of pig-to-non-human primate kidney xenotransplantation, now >125 days, provides promise for further study and potential clinical translation.

## Keywords

$\alpha$ -1,3-galactosyltransferase; costimulation blockade; human decay-accelerating factor; non-human primate; renal transplantation; transgenic pigs; xenoantigen; xenotransplantation

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## Introduction

Kidney transplantation is the treatment of choice for most individuals with impending or established end-stage renal disease. A major barrier to transplantation is the long-standing and ever-increasing disparity between the number of individuals listed for transplantation and the number of available organs. Xenotransplantation using pig organs has been proposed as a potential solution to the organ supply shortage [1,2]. Early attempts at xenotransplantation revealed the formidable immune barrier that exists between species, specifically hyperacute rejection resulting from natural preformed antibodies. One of the most important xenoantigens identified was galactose- $\alpha$ 1,3-galactose ( $\alpha$ Gal), which is expressed by pigs but not Old World primates and humans. The development of pigs genetically altered to lack  $\alpha$ Gal expression or engineered to transgenically express human complement- and thrombo-regulatory proteins has significantly improved but not eliminated the barrier to successful xenotransplantation [3,4]. Recent reports have detailed prolonged survival in a pig-to-baboon heterotopic heart model using antibody therapy targeting CD154 with one animal surviving longer than 1 yr [5]. Interestingly, progress in pig-to-monkey kidney xenotransplantation, where the graft is life sustaining, has been less impressive with median survival times of a few weeks and the longest reported survival of a single animal at 90 days [6]. Recipients frequently develop thrombotic microangiopathy and an accompanying consumptive coagulopathy characterized by severe thrombocytopenia [7]. Presumably this pathology is mediated by preformed, natural anti-pig antibody binding to the renal endothelium with resultant complement and coagulation cascade activation [8–11]. Because preformed antibodies are an important contributor to xenograft rejection, we screened a cohort of rhesus macaques for anti-pig antibody and selected potential recipients with both low and high titers. Here, we present our preliminary findings including the long-term survival (>125 days) of pig-to-non-human primate (NHP) kidney transplants using  $\alpha$ Gal knockout/CD55 transgenic pigs and an anti-CD154-based immunosuppressive protocol.

## Material and methods

### Animals

Four  $\alpha$ Gal knockout/CD55 transgenic pigs ( $\alpha$ -1,3-galactosyltransferase knockout, human decay-accelerating factor transgenic), aged 10–14 weeks, were obtained from the National Swine Resource and Research Center (University of Missouri-Columbia, Columbia, MO, USA) to serve as kidney donors. Five rhesus macaques were selected as renal transplant

recipients based on preformed IgG serum antibody levels against porcine  $\alpha$ Gal knockout cells, as described below.

### Pre-transplant serum antibody screening

Blood samples were collected from cloned  $\alpha$ Gal knockout pigs and separated using Ficoll-Paque Plus to collect peripheral blood mononuclear cells (PBMCs). Serum samples from 34 rhesus macaque transplant candidates were incubated with porcine  $\alpha$ Gal knockout PBMCs and then stained with anti-human IgM or anti-human IgG conjugated to Alexa-488 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Results were collected on an Accuri C6 flow cytometer with analysis of antibody binding by FlowJo version 8.8.7 (Treestar Inc., Ashland, OR, USA). Flow cytometry results were reported as molecules of equivalent soluble fluorochrome (MESF), and animals were ranked according to IgG and IgM preformed antibody titers. Four primates with the lowest preformed IgG titers and one macaque with the highest IgG titers were selected for transplantation.

### Pig-to-primate renal transplantation

Galactose- $\alpha$ 1,3-galactose knockout/CD55 transgenic pigs underwent unilateral or bilateral nephrectomy on the day of transplantation (Division of Animal Resources, Emory University, Atlanta, GA, USA). Rhesus macaques then underwent bilateral nephrectomy followed by life-sustaining renal transplantation with a donor porcine kidney (Yerkes National Primate Research Center, Emory University, Atlanta, GA, USA). Surgeries were performed in accordance with Institutional Animal Care and Use Committee regulations. Postoperatively, graft function was monitored with daily urine output assessment and weekly serum chemistries. Peripheral blood was collected for weekly cell subset analysis using flow cytometry. Quantitative cytomegalovirus (CMV) titers were measured weekly using a polymerase chain reaction (PCR)-based assay. Ultrasound-guided renal biopsies were performed postoperatively at days 14, 35, 70, and 100. Biopsy specimens were submitted for hematoxylin and eosin staining. Histologic analysis was performed by a blinded renal pathologist. Endpoint criteria for euthanasia were defined as serum creatinine  $>5$  mg/dl or blood urea nitrogen  $>100$  mg/dl on two consecutive measurements.

### Treatment regimen

Monkeys underwent T-cell depletion using anti-CD4 and anti-CD8 mAb plus costimulation blockade, either anti-CD154 or belatacept, and daily mycophenolate mofetil (MMF) and steroids (Table 1). T-cell depletion began 1–3 days prior to transplantation with a one-time dose of anti-CD4 50 mg/kg IV (clone CD4R1; NIH Nonhuman Primate Reagent Resource, Boston, MA, USA). Anti-CD8 (clone M-T807R1; NIH Nonhuman Primate Reagent Resource) was administered on postoperative day (POD) 0 at 50 mg/kg IV. Anti-CD154 (5c8; NIH Nonhuman Primate Reagent Resource) or belatacept (McKesson Medical, San Francisco, CA, USA) was given at 20 mg/kg IV on days 0, 7, and 14 then biweekly. Prophylactic enrofloxacin, fluconazole, and ganciclovir were started on the day of transplant. Fluconazole and enrofloxacin were discontinued when flow cytometry demonstrated sustained T-cell reconstitution. Ganciclovir was continued until CMV titers were negative on two consecutive measurements. Weekly subcutaneous Epogen injections

were started for hemoglobin less than 9.5 g/dl and discontinued when hemoglobin reached 12 g/dl.

### Flow cytometry

Peripheral blood was collected weekly to assess the effect of T-cell-depleting antibodies. Fluorescent mAbs against primate CD3 (SP34-2), CD4 (L200), and CD16 (3G8) were obtained from BD Biosciences (San Jose, CA, USA). Anti-human CD8 (DK25) was purchased from EMD Millipore (Billerica, MA, USA). Anti-primate CD19 (CB19) was acquired from Abcam (Cambridge, MA, USA). Samples were collected on a BD Biosciences LSR II flow cytometer and analyzed using FlowJo software, version 10.0.7 (Treestar Inc.).

### Cytomegalovirus assays

Porcine PBMCs, splenocytes, and lymphocytes derived from mesenteric lymph node were tested for porcine CMV by Zoologix, Inc. (Chatsworth, CA, USA) using real-time PCR. Primate PBMCs, renal biopsy samples, splenocytes, and/or lymph node-derived lymphocytes were also tested for porcine CMV. Fresh peripheral blood samples from monkeys were tested weekly for quantitative CMV viral loads using a primate-specific assay. Porcine PBMCs from two pigs were tested using the primate CMV assay to assess for cross-reactivity between assays.

### Statistical analyses

Post-transplant survival times were plotted and compared using Kaplan–Meier survival curves and log-rank tests with P-values <0.05 considered significant. All analyses were performed using Graph-Pad Prism, version 6.0 (La Jolla, CA, USA).

## Results

### Antibody titers and T-cell depletion

Serum samples from a cohort of 34 juvenile rhesus macaques of similar size and age were tested for anti-pig antibodies using a flow cytometric anti-pig cross-match assay (Figure S1). Based on these results, five recipient animals were selected to proceed with  $\alpha$ Gal knockout/CD55 transgenic pig-to-non-human primate kidney transplantation. Preformed anti-pig IgG antibody titers in the low-titer group of primates ranged from 1286 to 1599 MESF. IgM titers for the same group were 8410 to 20 517 MESF. IgG and IgM titers for the sole high-titer animal were 23 478 and 37 016 MESF, respectively (Fig. 1). All animals received similar immunosuppression consisting of the following: (i) T-cell depletion using a single dose of anti-CD4 and anti-CD8 given just prior to transplant; (ii) costimulation blockade (either anti-CD154 mAb or belatacept); and (iii) daily MMF and steroid. Flow cytometric analysis of peripheral blood subsets demonstrated effective CD8<sup>+</sup> T-cell depletion in all animals with repopulation occurring around 60 days after transplant (Fig. 2A). Post-treatment CD4<sup>+</sup> T-cell counts were more variable, in that two of five animals displayed complete CD4<sup>+</sup> T-cell depletion while the remaining three animals retained detectable CD4<sup>+</sup> cells to varying degrees (Fig. 2B). In the two animals with effective CD4<sup>+</sup> depletion, there was slow reconstitution of this compartment over time.

## Costimulation blockade with anti-CD154, but not belatacept, promotes long-term survival in monkeys with low anti-pig antibody titers

Non-human primate recipients were selected based on their pre-transplant anti-pig antibody titers. Given the proven benefits of anti-CD154 therapy in both cellular and solid organ experimental xenograft models, we selected an initial immunosuppression regimen consisting of induction with T-cell-depleting mAb and maintenance immunosuppression with anti-CD154, MMF, and steroid. Both low and high anti-pig antibody titer animals underwent kidney xenotransplantation. Despite initial early graft function, the animal with a high titer of anti-pig antibody quickly rejected the xenograft within the first-week post-transplant (Fig. 3A, Table 1). Clinical rejection was accompanied by severe anemia (hemoglobin 4.7 g/dl) and thrombocytopenia (platelets  $4 \times 10^3/\mu\text{l}$ ). Pathology revealed severe interstitial hemorrhage and edema with minimal cellular infiltrate as well as other histologic findings consistent with acute antibody-mediated rejection, including C4d and IgG deposition (Fig. 4A–H). In contrast, animals with low levels of pre-transplant anti-pig IgG who were treated with an anti-CD154 containing immunosuppressive regimen continue to have preserved renal function, now >125 days post-transplant (Fig. 3A, Table 1). Protocol kidney xenograft biopsies from day 100 revealed preserved renal architecture and minimal cellular infiltrate without evidence of rejection (Fig. 4K).

Although several costimulation blockade reagents, including antibodies against CD40 and CD154, are currently at various stages of clinical development, belatacept, a mutated form of CTLA-4-Ig that blocks binding of CD80/86 to CD28 on T cells, remains the only reagent that is presently approved for use in transplant recipients. Accordingly, we tested whether treatment with belatacept instead of anti-CD154 would prolong kidney xenograft survival. Despite low titers of anti-pig antibodies prior to transplant, recipients treated with the belatacept-based regimen rejected their xenografts at 2- and 3-weeks post-transplant (Fig. 3A, Table 1). Histologic analysis revealed a mixture of features consistent with acute cellular and antibody-mediated rejection as well as evidence of thrombotic microangiopathy (Fig. 4I,J).

### Electrolyte and hematologic parameters

Previous reports have confirmed the ability of the pig kidney to support normal renal function in a non-human primate recipient. However, some alterations in electrolytes and hematologic parameters in the post-transplant period were detected [12,13]. We encountered similar findings in our cohort of transplanted animals. In animals with functioning grafts, creatinine and potassium levels remained within normal limits (Fig. 3A,B). Phosphorus levels slowly declined over time, while serum calcium levels correspondingly increased (Fig. 3C,D). PTH values were assessed in the long-term survivors and were appropriately suppressed (data not shown).

Previous studies have reported significant anemia in macaques following kidney xenotransplantation [13]. This was attributed to decreased activity of porcine erythropoietin in non-human primate recipients. We chose to preemptively treat animals with recombinant human erythropoietin (rhEpo) when hemoglobin levels dropped below 9.5 g/dl. Long-term survivors, supported with exogenous rhEpo, maintained stable hematologic values post-

transplant, while recipients that experienced early rejection developed significant anemia despite rhEpo therapy (Fig. 5A). Severe thrombocytopenia has been observed frequently following pig-to-non-human primate kidney xenotransplantation. Animals that experienced early rejection had a significant drop in their platelet counts post-transplant including one animal with severe thrombocytopenia (platelets  $4 \times 10^3/\mu\text{l}$ ). In contrast, animals with preserved xenograft function continue to have stable platelet counts over time (Fig. 5B).

### Cytomegalovirus viral reactivation

Recent reports have suggested that CMV, particularly the infection status of the donor pig, may be an important factor in determining post-xenotransplant outcomes [14,15]. All of the  $\alpha\text{Gal}$  knock out/CD55 transgenic pig donors used in this study tested positive for porcine CMV (pCMV, data not shown). As part of the post-transplant treatment regimen, all animals were placed on prophylactic ganciclovir therapy. Peripheral blood samples from all recipients were tested weekly for rhesus CMV (rhCMV) using a PCR-based assay previously described. Three of the five animals developed significant rhCMV viremia ( $>10,000$  copies/ml) requiring treatment (Fig. 6). This was most profound in macaques with significant T-cell depletion. As expected, rhCMV viral loads improved with T-cell reconstitution. Given the significant degree of viremia, tissue samples from xenograft recipients were then tested for detection of pCMV. A single renal xenograft biopsy specimen tested positive for pCMV. All other tissues from transplant recipients were negative for the presence of pCMV DNA (Table 2). Despite established pCMV infection in the donor pigs, we were able to achieve long-term xenograft survival using an anti-CD154-based immunosuppression regimen.

### Discussion

Despite the remarkable progress in the field of transplantation over the last few decades, the shortage of available organs continues to be a major limitation. The use of organs from other species has been a theoretical solution but has been fraught with challenges. Significant progress has been made in the field of xenotransplantation particularly with the advent of genetically engineered pigs. The elimination of  $\alpha\text{Gal}$  represented a major step forward in preventing hyperacute rejection. In addition, the ability to express human complement and thromboregulatory proteins in pigs was a critical step to improve the known dysregulation of both the complement and coagulation cascades that occurs following xenotransplantation. Notwithstanding these advances, there has been limited success in kidney xenotransplantation with average survival times of a few weeks and the longest recorded non-human primate recipient of a pig kidney surviving to 90 days [6,16,17]. Here, we describe the longest ongoing survival time ( $>125$  days) reported to date in a life-sustaining, pig-to-non-human primate renal xenograft model using an anti-CD154-based immunosuppression regimen.

While anti-Gal antibodies represent an important component of the humoral response to xenogeneic organs, it is now clear that there are additional pig antigens that are recognized by natural antibodies found in humans and primates and that these antibodies contribute to xenograft rejection [18]. These non-Gal antibodies are a major barrier to successful kidney



xenotransplantation. Similar to humans, the titers of natural anti-non-Gal antibodies in NHPs vary significantly between individuals. We tested a large cohort of animals for non-Gal antibody levels and then selected animals with low and high titers to proceed with xenotransplant. As expected the level of pre-transplant, non-Gal anti-pig antibody played an important role in determining post-transplant outcomes in our study. There are numerous pig antigens, likely glycoproteins or glycolipids, that serve as targets for preformed antibody despite the absence of  $\alpha$ Gal. These non-Gal antibodies have been detected in human and non-human primate serum and at least some of them can mediate complement-dependent lysis and antibody-dependent cellular cytotoxicity to pig cells [19,20]. Previous reports have identified two important glycosyltransferases, cytidine monophospho-*N*-acetylneuraminic acid hydroxylase (CMAH) and  $\beta$ -1,4 *N*-acetylgalactosaminyl transferase ( $\beta$ 4GalNT2), that contribute to the production of important xenoantigens in humans and non-human primates [21–24]. Selective deletion of these enzymes in combination with the enzyme responsible for  $\alpha$ Gal production, 1,3-galactosyl transferase, has the potential to lower the pre-transplant anti-pig antibody titers to a point where we might consistently achieve long-term kidney xenograft function in most if not all recipients when treated with an effective immunosuppression regimen [25–27].

In our study, high titers of anti-pig antibody pre-transplant resulted in an accelerated form of acute humoral xenograft rejection while low titers had variable results depending on the immunosuppression regimen. The two immunosuppression regimens only differed by the reagent used to block T-cell costimulation, either belatacept or anti-CD154. We selected belatacept as it is the only costimulation blockade reagent currently approved for use in transplantation. We have previously reported its efficacy in several non-human primate models of allotransplantation [28,29]. Despite having low titers of anti-pig antibody pre-transplant, xenograft recipients receiving belatacept underwent rejection early post-transplant (2 and 3 weeks). Histologically, there were features of both antibody and cellular rejection. Despite the protective effects of belatacept on renal function and cardiovascular risk, there is a significantly higher rate of rejection in belatacept-treated patients [30]. There may be several reasons why belatacept failed to control the xenogeneic response. Belatacept binds to both CD80 and CD86 and blocks their interactions with the T-cell costimulatory molecule CD28. This interaction also limits binding of CTLA-4 and the resultant coinhibitory signals [31]. This imbalance of not only costimulatory but also co-inhibitory signals may ultimately impact T-cell differentiation and function, particularly in certain T-cell subsets such as regulatory T cells that may be dependent on those coinhibitory signals. In addition for reasons that are unclear, the level of CD4<sup>+</sup> T-cell depletion in the animals treated with belatacept was less complete than those who were treated with anti-CD154.

Another potential reason for the lack of efficacy may be related to the development of belatacept. Point mutations were introduced to increase the binding activity of the parent molecule, CTLA-4-Ig, which resulted in increased binding affinity to human CD80 and CD86. Interestingly, these changes also eliminated the ability of belatacept to bind to the murine versions of CD80 and CD86. Similarly, there is also some question as to how well belatacept binds to porcine CD80 and CD86. This may provide a plausible explanation for its decreased efficacy [32]. Newer reagents have recently been developed to specifically target CD28 and potentially eliminate both of these concerns; permitting coinhibitory signals

through CTLA-4 and targeting CD28 directly which is expressed on the recipient T cells (human or non-human primate). We and others have shown promising results with these reagents in allotransplantation [33–35].

In contrast to the belatacept-treated cohort, animals with low titers of anti-pig antibody which received the anti-CD154 containing regimen continue to enjoy rejection-free survival now greater than 4 months post-transplant. Blockade of the CD40-CD154 pathway seems to be a critical component of most immunosuppression regimens that result in long-term survival of solid organ or cellular xenografts [36–38]. Previous attempts to introduce anti-CD154 therapies for clinical use were met with several challenges including an increased incidence of thromboembolic events. We and others have focused our efforts on the development of safer alternatives including antibodies to CD40 and novel domain antibodies targeting CD154 which are presumably devoid of the unwanted side effects seen with previous anti-CD154 therapies [39–41]. Some of these reagents are moving forward in clinical trials of autoimmunity and transplantation. The availability of such reagents in combination with novel pigs, genetically engineered to lower preformed xenoreactive antibodies to acceptable levels, could provide a means of advancing xenotransplantation from the experimental to a clinical reality.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>ACR</b>	acute cellular rejection
<b>AMR</b>	antibody-mediated rejection
<b>CMV</b>	cytomegalovirus
<b>Hgb</b>	hemoglobin
<b>mAbs</b>	monoclonal antibodies
<b>MESF</b>	molecules of equivalent soluble fluorochrome
<b>MMF</b>	mycophenolate mofetil
<b>PBMCs</b>	peripheral blood mononuclear cells
<b>PCR</b>	polymerase chain reaction
<b>POD</b>	postoperative day
<b>PTH</b>	parathyroid hormone

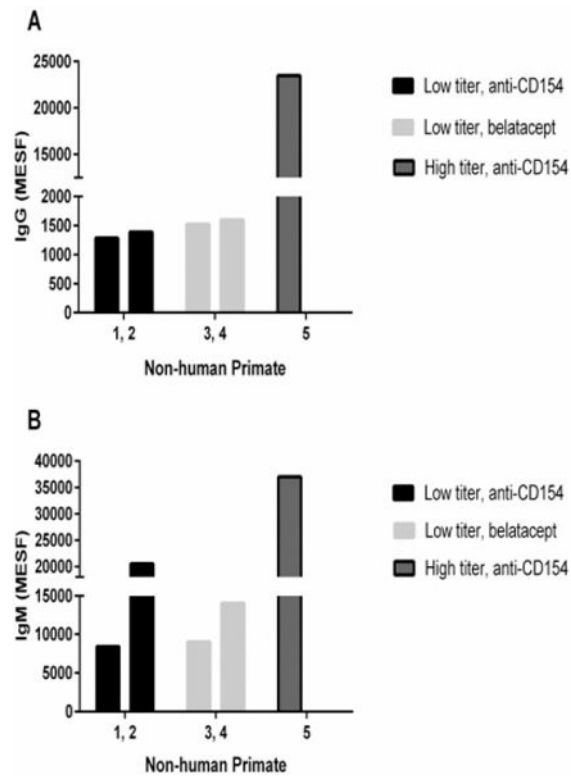


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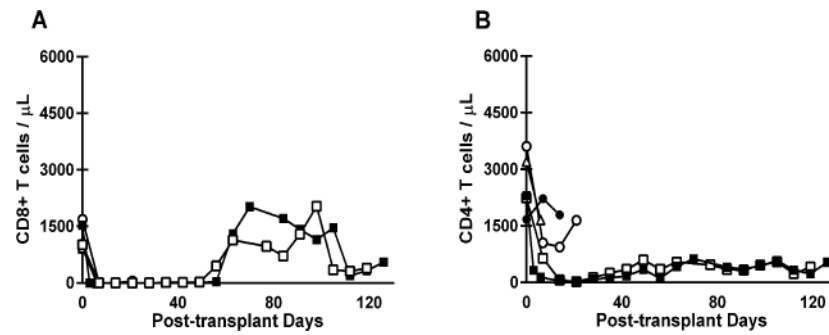
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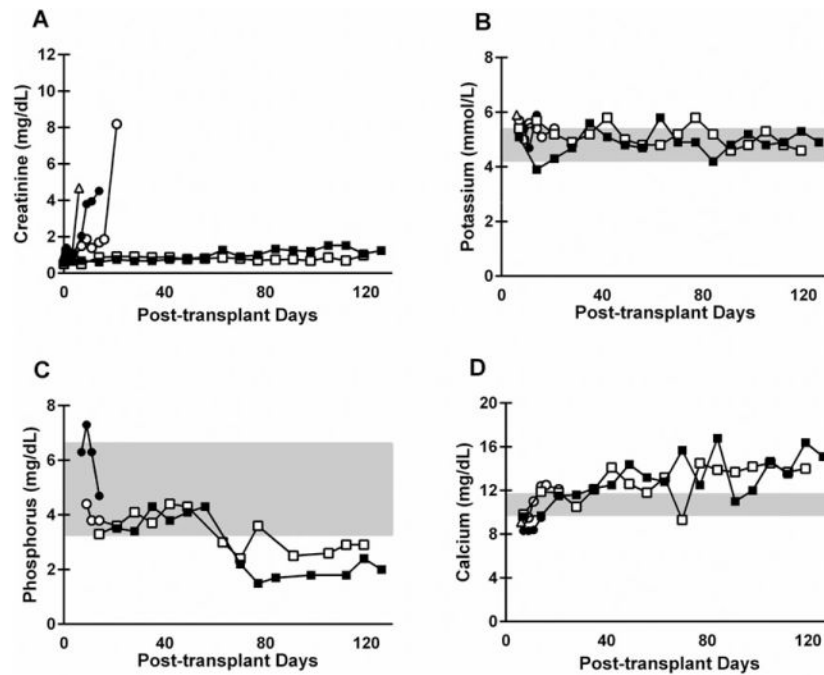


**Fig. 1.** Pre-transplant antibody titers against galactose- $\alpha$ 1,3-galactose ( $\alpha$ Gal) knockout pig cells. Thirty-four non-transplanted rhesus macaques were screened for IgG and IgM antibody titers against porcine  $\alpha$ Gal knockout peripheral blood mononuclear cells. (A) Four monkeys with the lowest IgG titers were selected to receive a porcine  $\alpha$ Gal knockout/CD55 transgenic renal xenograft. One monkey with the highest IgG titer was also selected. (B) IgM levels varied across treatment group.



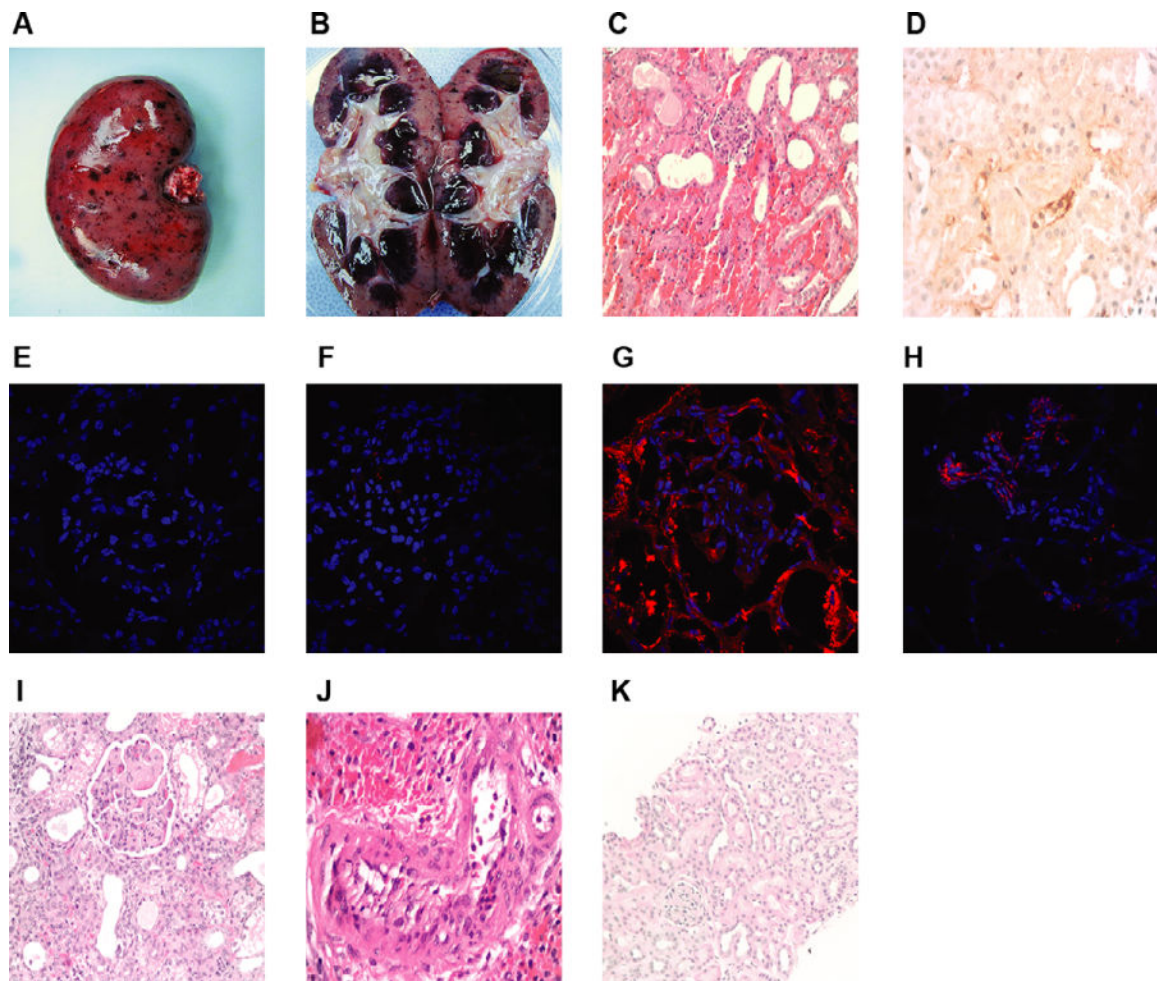
**Fig. 2.**

Flow cytometric analysis of peripheral blood in animals receiving T-cell depletion. Peripheral blood immunophenotypic analysis was performed using flow cytometry. Baseline total T-cell, CD8<sup>+</sup> T-cell, and CD4<sup>+</sup> T-cell counts were measured prior to administration of T-cell-depleting antibodies. After xenotransplantation, weekly flow cytometry was performed to monitor for reconstitution of T-cell populations. Low-titer, anti-CD154 monkeys are represented by open and filled squares. Low-titer, belatacept monkeys are depicted as open and filled circles. High-titer, anti-CD154 monkey is shown as open triangles. (A) Successful CD8<sup>+</sup> T-cell depletion was achieved in each monkey with full reconstitution occurring after POD 60. (B) Variable CD4<sup>+</sup> T-cell depletion was observed among animals, with slow reconstitution of this population over time.



**Fig. 3.** Serum creatinine and electrolyte levels after pig-to-primate renal xenotransplantation. Serum chemistries were obtained on a weekly basis post-transplant to monitor graft function. Shading depicts normal electrolyte ranges for rhesus macaques. Low-titer, anti-CD154 monkeys are represented by open and filled squares. Low-titer, belatacept monkeys are depicted as open and filled circles. High-titer, anti-CD154 monkey is shown as open triangles. (A) In monkeys with functioning grafts, creatinine values remained within the expected range. (B) Potassium levels remained constant and within normal limits post-transplant. (C) Phosphorus decreased over time, most notably after postoperative day 60. (D) Calcium levels increased after transplant with levels slightly higher than the anticipated range for macaques.



**Fig. 4.**

Gross appearance, histology, and immunofluorescence of pig-to-primate renal xenografts. Protocol renal biopsies were obtained at postoperative day (POD) 14, 35, 70, and 100. At rejection, animals underwent transplant nephrectomy with subsequent histologic analysis. (A, B) Animals that rejected early (POD 6–21) demonstrated gross congestion and hemorrhage in the xenograft at time of rejection. (C, D) H&E staining of rejected renal xenograft in the high-titer monkey demonstrated interstitial hemorrhage and edema. C4d staining was positive, consistent with antibody-mediated rejection. (E–H) Immunofluorescence in the high-titer monkey showed significant deposition of IgG, but not IgM, in the rejected xenograft. Fixed porcine tissue was stained with DAPI (blue) and AF647-anti-IgG or AF647-anti-IgM (red). (E) IgG and (F) IgM staining in a control non-transplanted  $\alpha$ Gal knockout porcine kidney showed no antibody deposition. (G) Substantial IgG binding was seen in the transplanted renal xenograft at the time of rejection. (H) Minimal IgM staining was seen in the same rejected xenograft. (I, J) H&E staining of rejected xenograft in low-titer, belatacept-treated monkeys showed evidence of antibody-mediated rejection and acute cellular rejection. Both thrombotic microangiopathy (I) and arteritis (J) were seen. (K) H&E staining of protocol renal biopsies in low-titer, anti-CD154-

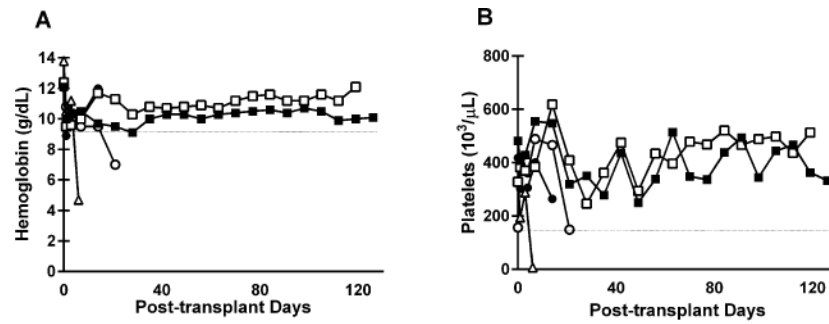
treated monkeys demonstrated preserved renal architecture and no evidence of rejection at POD 100.

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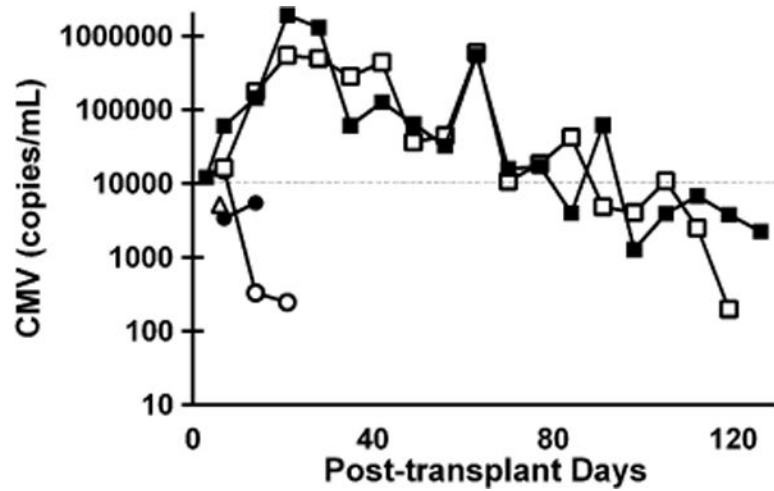
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**Fig. 5.**

Hemoglobin and platelet measurements after pig-to-primate renal xenotransplantation.

Hemoglobin and platelet counts were monitored on a weekly basis. (A) All monkeys started exogenous rhEpo when hemoglobin dropped below 9.5 g/dl. Time of rhEpo initiation ranged from postoperative day 1–20. Two of three animals with histology-proven rejection developed severe anemia at time of rejection. Animals with functioning grafts maintained normal hemoglobin values. (B) Platelet counts remained stable over time in animals without clinical or histologic evidence of rejection. Two animals with antibody-mediated rejection developed significant thrombocytopenia at time of rejection.



**Fig. 6.** Cytomegalovirus (CMV) titers following T-cell depletion and costimulation blockade. CMV titers were measured weekly using polymerase chain reaction. Standard cutoff for initiation of antiviral therapy is greater than 10 000 copies/ml. However, given the concern for CMV viremia in the setting of T-cell depletion, all monkeys were started on prophylactic ganciclovir beginning the day of transplant. Despite this, two monkeys with the greatest T-cell depletion developed significant CMV viremia. As T-cell counts recovered, CMV titers improved.

**Table 1**

Treatment and survival times following pig-to-primate renal xenotransplantation. Monkeys were divided into three treatment arms: (i) low pre-transplant anti-pig titers and anti-CD154 costimulatory blockade; (ii) low titer and belatacept; and (iii) high titer and anti-CD154. Survival ranged from 14 to >133 days in low-titer monkeys with 6-day survival in the high-titer monkey. Among the low-titer group, improved survival was observed in animals treated with anti-CD154

<b>NHP</b>	<b>Pre-transplant, anti-pig Ab titers</b>	<b>Therapy</b>	<b>Survival (days)</b>
#1	Low titer	Anti-CD4, anti-CD8, anti-CD154, MMF/steroids	>133
#2	Low titer	Anti-CD4, anti-CD8, anti-CD154, MMF/steroids	>126
#3	Low titer	Anti-CD4, anti-CD8, belatacept, MMF/steroids	14
#4	Low titer	Anti-CD4, anti-CD8, belatacept, MMF/steroids	21
#5	High titer	Anti-CD4, anti-CD8, anti-CD154, MMF/steroids	6

**Table 2**

Non-human primate tissues tested for porcine cytomegalovirus (CMV) using porcine-specific CMV assay. Tissue from xenotransplant recipients was tested for porcine CMV given the significant degree of CMV viral loads detected in the peripheral blood. Only one transplanted xenograft tested positive for porcine CMV

<b>NHP</b>	<b>Tissue(s)</b>	<b>Porcine CMV assay</b>
#1	Kidney	Neg
#3	Kidney	Neg
	Lymph node	Neg
	Spleen	Neg
	PBMC	Neg
#4	Kidney	Pos
#5	Kidney	Neg
	Lymph node	Neg
	PBMC	Neg

PBMC, peripheral blood mononuclear cells.