Progressive Genetic Modifications with Growth Hormone Receptor Knockout Extends Cardiac Xenograft Survival to 9 Months

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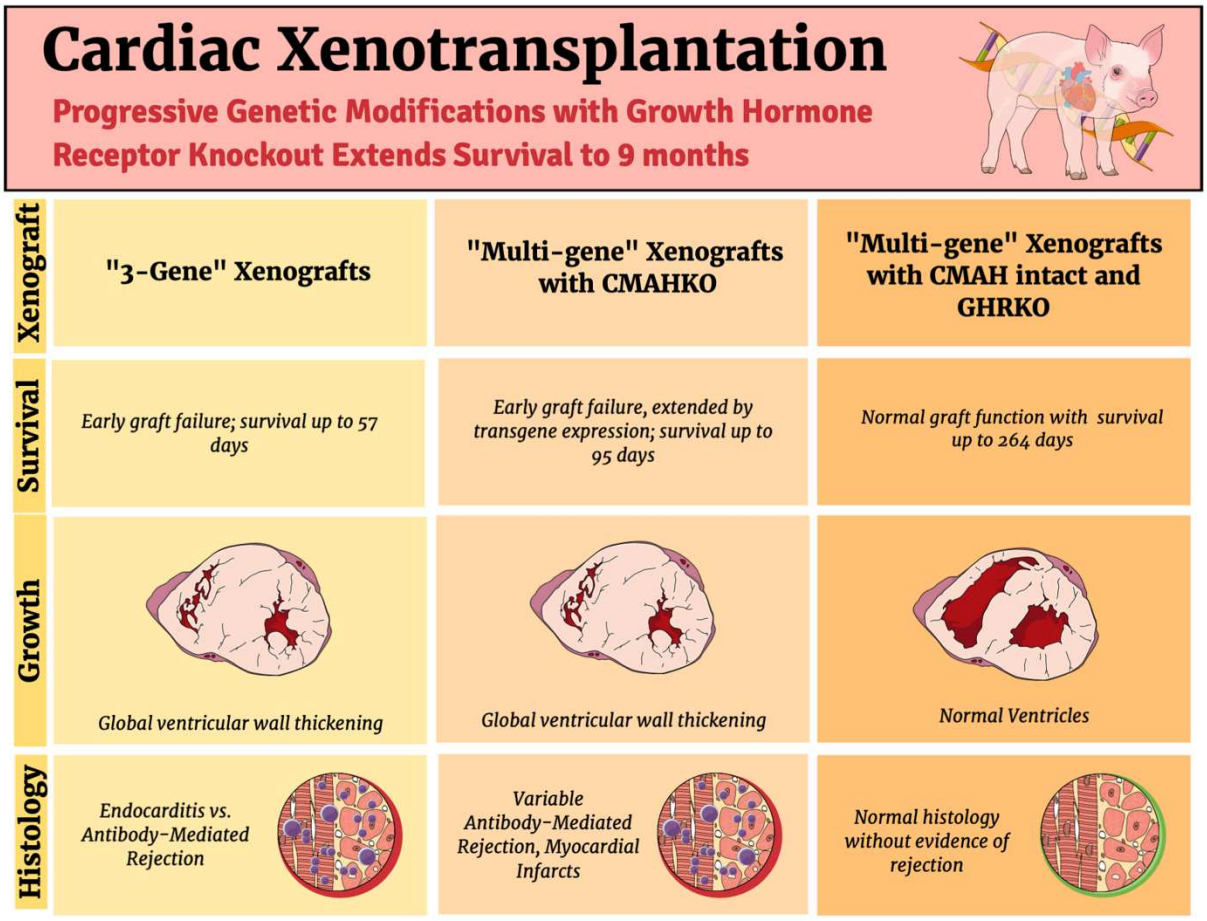

Abstract:

We report orthotopic (life-supporting) survival of genetically engineered porcine cardiac xenografts (with 3-9 progressive gene modifications) for almost 9 months in baboon recipients. This work builds on our previously reported heterotopic cardiac xenograft (3 gene modifications) survival up to 945 days with an anti-CD40 monoclonal antibody-based immunosuppression. In this current study, life-supporting xenografts containing multiple human complement regulatory, thromboregulatory, and anti-inflammatory proteins, in addition to growth hormone receptor knockout (KO) and carbohydrate antigen KOs, were transplanted. Selective "multi-gene" xenografts demonstrate survival greater than 8 months without the use of adjunctive medications and without evidence of abnormal xenograft thickness or rejection. These data demonstrate that selective “multi-gene" modifications improve cardiac xenograft survival significantly and may be foundational for paving the way to bridge transplantation in humans.

Introduction:

We have reported previously almost three-year survival of genetically engineered (GE) pig cardiac xenografts in an abdominal, non-load bearing heterotopic heart transplantation (HHTx) in baboons. Initial gene modifications in this model targeted hyperacute antibody-mediated rejection and dysregulation of thrombosis, which included deletion of a carbohydrate antigen (α1,3-galactose) and insertion of human complement inhibitory protein (hCD46) along with human thrombomodulin, an inhibitor of thrombin (hTBM). We demonstrated the use of a non-depleting, anti-CD40 (2C10R4) monoclonal antibody (mAb)-based immunosuppressive regimen

("Mohiuddin Regimen") that led to rejection-free survival of 945 days[1], which has been reliably reproduced.[2–6]

Our initial efforts to translate these results in a life-sustaining orthotopic heart transplantation (OHTx) failed within 48 hours due to perioperative cardiac xenograft dysfunction (PCXD).[7] Recently, Längin, *et al*. overcame PCXD in OHTx with the same GE xenografts and immunosuppression as in our HHTx, with the addition of non-ischemic cardiac preservation and anti-inflammatory agents.[3] However, survival was still limited due to observed diastolic failure from abnormal cardiac growth within one month. This post-transplantation xenograft growth is poorly understood, but six-month recipient survival was achieved after the additional administration of temsirolimus (inhibitor of growth by mTOR, but also an immunosuppressive agent) along with strict blood pressure and heart rate control to prevent this growth. Withdrawing temsirolimus resulted in continuation of growth and ultimately xenograft failure. Recently, we observed that this growth is independent of heart rate and afterload reduction, suggesting an etiology separate from physiologic mismatch.[8]

Xenograft rejection is a result of endothelial cell activation by preformed antibodies against porcine antigens.[9,10] This leads to hyperacute rejection within minutes of transplantation, characterized by endothelial damage, antibody deposition, complement activation and intravascular thrombosis.[11] However, several scientific discoveries have led to the ability to produce targeted modifications to porcine xenograft donors that reduce their immunogenicity and abrogate this reaction. Knockout of xenogeneic carbohydrate antigens α1,3-galactose (Gal), SDa blood group antigen (SDa) and N-glycolylneuraminic acid (Neu5Gc) have been shown to

reduce antibody binding and complement-dependent cytotoxicity against non-human primates and human sera alone and in combination.[12] Rejection is further prevented by the additional expression of human complement regulatory proteins hCD46 and decay-accelerating factor (hDAF).[13,14] Thrombotic microangiopathy and consumptive coagulopathy also play a role in rejection, possibly due to incompatible factors of thrombosis between porcine endothelial cells and recipient serum.[15] Factors that promote anti-coagulation, such as human thrombomodulin (hTBM) have been shown to prevent coagulation dysregulation and survival.[1,3,16,17] Human anti-inflammatory (i.e. heme oxygenase-1 (hHO-1)) and anti-phagocytosis genes (i.e. hCD47), respectively, have also demonstrated a role in abrogating transplant rejection *in vitro*.[18–20] Only recently, advances in genetic engineering afforded the opportunity to produce porcine donors with all of these targeted modifications in combination.[21,22]

In this study, we examine the utility of "multi-gene" xenografts up to 9 genetic modifications from GE pigs. We also chronicle our experience with xenografts of less than 9-gene modifications, as the limited production of these pigs and very expensive transplant procedures, limits our ability to test every gene iteration, but still provides an opportunity to evaluate several genetic constructs of less than 9-genes.

All baboon recipients were treated with our previously described anti-CD40-based IS regimen and transplanted with a life-supporting cardiac xenograft from a GE pig without additional immunosuppression or drugs to control cardiac growth. We then examine which "multi-gene" xenograft, in combination with our CD-40 mAb-based regimen, might produce survival conducive for initial human clinical trials.

Results:

Genetically engineered pigs with relevant gene editing provide less immunogenic donor hearts for transplantation:

Correct, single copy targeting of transgene constructs to landing pads was confirmed by PCR, Southern blot and digital drop PCR. Knockout (KO) of α-1,3-galactosyltransferase (GGTA1, the enzyme responsible for synthesis of Gal) was confirmed by PCR for presence of a disruptive NeoR insertion in exon 9.[23] KO of genes encoding β1,4-N-acetylgalactosyltransferase (β4GalNT2, the enzyme responsible for synthesis of SDa), CMP-N-acetylneuraminic acid hydroxylase (CMAH, the enzyme responsible for synthesis of Neu5Gc) and growth hormone receptor (GHR) were assessed by Next-Gen DNA sequencing (MiSeq, Illumina) for the presence of large or frameshifting indels. Phenotypes of GGTA1KO, B4GALNT2KO and CMAHKO knockout were confirmed by flow cytometry of PBMC stained with IB4 lectin, DBA lectin and anti-Neu5Gc respectively, to reveal absence of xenogeneic carbohydrate residues catalyzed by the knocked-out gene product (figure 2a-c). GHRKO phenotype was determined by demonstrating reduced serum IGF-1 levels (figure 2d) and body weight[24] at 142 days of age (65.77±9.17 kg vs. 32.27±1.20 kg for GHR wild-type and GHRKO pigs, respectively; Mean±SD; $P<0.01$). Expression of individual transgenes was confirmed in tail biopsies of donor pigs prior to transplantation by western blot (figure 2e) and demonstrated continued expression in explanted heart tissues by immunohistochemical staining (figure 2f).

Multiple gene edits reduced the immunogenicity of cardiac xenografts as measured by antibody binding and complement-dependent cytotoxicity assays. Porcine aortic endothelial cells (pAECs) from OHTx pig donors with double carbohydrate KO (GGTA1KO and β4GalNT2KO) had reduced binding, compared to single carbohydrate KO (GGTA1KO), but paradoxically worsened with triple carbohydrate KO xenografts (GGTA1KO, β4GalNT2KO and CMAHKO) (figure 3a-b). CMAHKO (-/+) and CMAHKO (-/-) pAECs showed similar total IgM MFI by flow cytometry (figure 3c), despite CMAHKO (-/-) having reduced anti-Neu5Gc staining by flow cytometry (figure 2b). Group 3 xenografts produced the least amount of complement-dependent cytotoxicity (CDC), followed by Group 4 xenografts. CDC was elevated in Groups 1 and 2 and was markedly reduced with the addition of hCD46 and hDAF (figure 3d-e).

Consistent long-term survival of cardiac xenografts by progressive elimination of immunogenic pig antigens and expression of human transgenes

All recipients were easily weaned from cardiopulmonary bypass (supplementary table 1) after successful life supporting OHTx from GE pigs with 3-9 gene modifications. Improved survival with progressive genetic modifications is summarized in figure 4. Group 1 xenografts, with the gene construct similar to one used in our HHTx model (i.e., GGTA1KO, hTBM, and hCD46 (n=4)), survived up to 57 days (mean=23.5±25.3). Single KO or TKO xenografts in Group 2, without thromboregulatory proteins failed within 1 week. Survival did not improve despite the addition of complement regulatory proteins (CRP; hCD46 and hDAF) (Group 2, n=4).

GE pigs with nine gene modifications (GGTA1KO, B4GalNT2, CMAHKO, hTBM, hEPCR, hCD46, hDAF, hCD47, hHO1, referred to as Group 3) or seven gene modifications (GGTA1KO, B4GalNT2, hTBM, hEPCR, hCD46, hCD47 and GHRKO, referred to as Group 4) demonstrated significant prolongation of xenograft survival. Cardiac xenograft survival in Groups 3 (n=2) was extended to a mean of 89.5±7.8 days. In Group 4 (n=2), xenograft survival was markedly prolonged to a mean of 223±58.0 days. One xenograft functioned for 264 days prior to explantation (B33130), which is the longest reported life supporting xenograft survival to date. The other recipient (B32863) from Group 4 demonstrated reduced food intake from gingivitis and had to be euthanized on postoperative day (POD) #182 for excessive weight loss as per our institution's protocol. Overall, cardiac xenograft function was excellent on transthoracic echocardiography (TTE) at the time of euthanasia (video 1).

Elimination of GHR gene from donor GE pigs resulted in cardiac xenografts with reduced growth and improved survival

To address intrinsic post-transplantation xenograft growth, pig donors' GHR was knocked out in addition to the other genetic modifications as previously mentioned. As in Groups 1 and 2, adjuncts to reduce post-transplantation xenograft growth (temsirolimus and heart rate and afterload reducers) were not employed.

All xenografts from Groups 3 and 4 exhibited preserved systolic function post-transplantation and survival measured in months, but Group 3 xenografts (which have intact GHR) developed lower extremity edema, dyspnea, and lethargy by POD #84 and 95 and were subsequently

euthanized. On TTE, significant ventricular wall thickening was observed. Cross-sections of the explanted hearts confirmed biventricular wall thickening (figure 5a). Thus, ventricular wall thickening in Group 3 was delayed until POD #55, with additional gene modifications, but ultimately not prevented. Group 4 xenografts, that also lack functional growth hormone receptor (GHRKO), continued to function without myocardial thickening on TTE for up to 264 days prior to explantation. No apparent xenograft thickness was seen on cross-sections of the explanted xenograft from this group either (figure 5b). The explanted xenograft's gross appearance was similar to a naive pig heart. Mean arterial pressures (MAP) and heart rates were elevated compared to pig's native ranges in Groups 3 and 4. Despite the presence of this physiologic mismatch, and the absence of treatment, significant graft growth was not observed in Group 4.

Histopathological and immunohistochemical evaluation of xenografts demonstrates the advantage of multigene modifications of the cardiac xenograft

In Group 1 xenografts, with survival passed 48 hours (and thus surpassed PCXD), endocarditis, monocyte and neutrophil infiltration, and fibrin thrombi was demonstrated (supplemental figure 1). The presence of rejection versus an inflammatory process was difficult to interpret, but histologic and IHC examination, along with non-gal antibody titer elevation suggested AMR (B32628). Thrombotic complications were seen in 50% of xenografts without expression of thromboregulatory proteins (n=4, Group 2). On gross postmortem examination, notable intracardiac thrombi were seen with propagation into the aorta, pulmonary arteries, and coronary sinus of some of these xenografts (figure 6a). Histologic examination revealed intracardiac

organizing thrombus, intravascular fibrin thrombi with regions of myocardial ischemia (figure 6b).

Group 3, with xenografts containing CMAHKO, showed evidence of rejection within 3 months, with the progressive addition of human transgenes improving survival (figure 4). Compared to CMAHKO grafts in Group 2, xenograft survival was significantly prolonged, but ultimately recipients were euthanized for symptoms developed in the presence of myocardial thickening. Gross examination of these explanted xenografts demonstrated ventricular wall thickening, as mentioned previously (figure 5a). Space-filling inflammatory changes, edema, and fibrosis were present in those xenografts with wall thickening (supplementary figure 2). There was no histologic evidence of frank hypertrophy, that is characterized by myocardial disarray, hypertrophy of myofibers, or irregular branching of cardiomyocytes. Although there was scattered evidence of anti-pig antibody deposition in other groups (supplemental figure 4a and 4b), with less C4d staining with transgenes' expression, evidence of antibody-mediated rejection (AMR) was dominant only in Group 3 xenografts. These findings include interstitial edema and interstitial hemorrhage, microvascular thrombosis, fibrosis, cellular infiltration, and endotheliitis (figure 7a-7b). The other Group 3 xenograft contained mild interstitial inflammation and chronic xenograft vasculopathy (figure 7c-d). Notably, however, a large acute septal infarct was present (supplemental figure 2g).

Group 4 xenografts consistently produced survival greater than 6 months, without evidence of growth, rejection or inflammation. One (B32863) of the two recipients was euthanized on POD# 182 due to weight loss and demonstrated normal histology without evidence of rejection or

inflammation (figure 7e and f). Endomyocardial biopsy was performed on the other long-term survivor (B33130) on POD# 220, and H&E staining also demonstrated normal histology (figure 7g and h). Terminal histology of this xenograft on POD# 264 demonstrated evidence of chronic vasculopathy of smaller vessels leading to xenograft failure. These xenografts demonstrate excellent biventricular function and minimal graft thickening, even in B33130 on POD# 260, just 4 days prior to explantation (video 2).

Quantification of C4d, IgM, IgG staining and vascular microthrombi are summarized in figure 8 and supplementary figure 4a and 4b. Group 4 had the most favorable IHC staining, as demonstrated by minimal C4d, IgM, IgG and microthrombi staining, followed by Group 1. C4d deposition was minimal in Groups 1 and 4, whereas IgM and IgG deposition was minimal only in Group 4. Xenografts containing CMAHKO (Groups 2 and 3) had the least favorable IHC staining with relatively high levels of both IgM, IgG staining and C4d staining.

Immunologic analysis in long-term versus short-term survivors:

Immunophenotyping was performed on PBMCs from recipients longitudinally after transplantation. T and B-cell lymphocytes were depleted with ATG and Rituxan and confirmed by flow cytometry. CD3+ lymphocytes reconstituted within 48 hours of transplantation. However, CD20+ lymphocyte depletion was for a prolonged period. In B33121, CD20+ lymphocytes re-emerged by 60 days; otherwise, all other long-term recipients reconstituted by 120 days, consistent with previous studies by our group (figure 9).[1,25]

Serum cytokine levels were measured after xenotransplantation in all the recipients receiving cardiac xenograft from all 4 groups. There were no observed differences in peripheral cytokine levels between short and long-term survivors or those with anti-inflammatory genes (Group 3, HO-1) (supplementary figure 3). Additionally, circulating anti-pig non-gal IgG and IgM antibody levels did not rise significantly in any xenografts with early graft failure, even after AMR (supplementary figure 5). There were no trends found between pretransplant non-gal IgG or IgM antibody levels and xenograft deposition postmortem.

Laboratory results demonstrate lasting protection from rejection in xenografts with multiple knockouts and human complement, thrombosis, and inflammatory regulatory proteins

Complete blood count (CBC), complete metabolic profile (CMP), coagulation parameters, and troponin I were collected at regular time intervals throughout the study (supplemental figures 6-9). Long-term survivors in Group 3 and 4 maintained normal kidney and liver function, whereas short-term survivors' end-organ function was more variable (supplemental figure 7).

Troponin levels peaked immediately after transplantation, consistent with ischemia/reperfusion insults on the xenograft followed by resolution down to 0.01-0.08 ng/mL out to 90 days, with a new baseline troponin between 0.10-0.94 ng/mL in those functioning past 3 months (i.e., Group 4) (supplemental figure 8). Most notably, troponin I correlated with rejection prior to xenograft dysfunction and resulted in troponin levels well over 1.0 ng/mL. Moreover, troponin was found to spike to levels well over baseline levels in the context of recipient stress, such as during

central line associated infections or examination under sedation and were unrelated to rejection episodes. After inciting events, troponin levels trended back to baseline.

Partial thromboplastin time (PTT), a measure of heparin anti-coagulation levels, when targeting an activating clotting time (ACT) greater than twice baseline levels, was almost always >100 seconds. As seen in supplemental figure 9, all long-term survivors achieved therapeutic anti-coagulation to these levels but had periodic reductions in PTT. During these levels of relatively less anti-coagulation, troponin levels did not rise, and D-dimer, fibrinogen and platelet levels remained normal.

Discussion:

Xenograft rejection is complex, and its mechanism appears much different from that of allografts. While allografts are predominantly rejected via cell-mediated mechanisms, xenografts are afflicted by a robust antibody-mediated process. Complex incompatibilities exist between donor pig and recipient non-human primates (and humans), which activates complement and induces dysregulation of coagulation.[10] To date, AMR from preformed or induced antibodies to 3 carbohydrate antigens have been identified (Gal, SDa, and Neu5Gc).[26–28] These factors have been addressed with immunosuppression, gene knockout and pigs expressing human transgenes developed in HHTx models of transplantation.[1,29] As HHTx models were transitioned to OHTx, PCXD became a significant barrier but has now been mitigated with improved preservation of the donor organ. The longer-term survival, afforded by overcoming PCXD in OHTx, allows the investigation of various genetic modifications in life-supporting cardiac xenografts.

We have shown a stepwise increase in xenograft survival by addressing the main contributing factors of immunologic rejection with additional knockouts and expression of human transgenes in pig donors. Select multi-gene xenografts demonstrate function for up to 264 days and exhibit minimal post-transplantation xenograft growth. Physiologic mismatch between pig xenograft and recipient baboon have not been shown to play a significant role in early graft failure. In our series, thickening was significantly delayed by additional transgene expression and KOs in Group 3 and was avoided in Group 4, and did not require afterload reduction, heart rate control, or Temsirolimus administration as previously suggested.[30] The histological evidence presented in this study supports the idea that this growth, while multi-factorial, may have a rejection component that contributes significantly to ventricular wall thickness, xenograft dysfunction, and recipient demise. Temsirolimus has been used to mitigate "growth." While an inhibitor of mTOR and intrinsic graft growth, is also an immunosuppressive agent, and perhaps its role in suppressing graft growth is dependent on its anti-rejection effect. Indeed, in HHTx, we have not observed post-transplantation cardiac xenograft growth until rejection occurs from removing anti-CD-40 mAb immunosuppression.[1] In this study, intact GHR signaling in xenografts likely also contributes to post-transplantation growth in Group 3 (non GHRKO xenografts) compared to Group 4 (GHRKO xenografts). A multi-factorial working model for post-transplantation xenograft growth is depicted in figure 10.

Paradoxically, AMR continues to play a significant role in the non-human primate model with xenograft KO of Neu5Gc.[27] Immunologic and histopathologic analysis is consistent with CMAHKO-related *de novo* neoantigen production in Groups 2 and 3, which may be driving

AMR. This is also supported by demonstrating that CMAHKO has increased IgM binding compared to double KO xenografts (figure 3c). As shown in figure 2c, while heterozygous KO of CMAH is phenotypically null for Neu5Gc, these xenografts still have increased IgM binding compared to double KO xenografts (figure 3c). However, this is purely a limitation of the NHP model, as humans already have a functional mutation of CMAH, which prevents B/T cell education of this antigen in humans and are tolerant to this potential neoantigen.[31] Thus, xenografts with CMAHKO may not show accelerated rejection when transplanted in human recipients.[32] While additional transgenes protected xenografts containing CMAHKO and delay graft failure, these constructs do not completely prevent it (Group 2 vs. 3).

This study also reveals the potential additive benefit of other transgenes in addition to GHRKO. Specifically, hTBM, EPCR, hCD46 and hDAF. Survival was increased in xenografts that contained hTBM compared to grafts that did not. In xenografts that did not contain thromboregulatory proteins, thrombotic complications were present. This suggests that hTBM and other thromboregulatory proteins such as EPCR are equally important to xenograft protection and survival as immunomodulatory proteins. It is already known that there is an incompatibility between porcine-derived thrombomodulin from the endothelium to non-porcine circulating thrombin, and this contributes to the complex xenotransplantation rejection process. However, its role in preventing thrombosis in an OHTx has not been demonstrated yet. We also demonstrate a markedly reduced CDC with the addition of hCD46 expression with an additive benefit in combination with hDAF. This translated to a significant survival benefit when combined with xenografts containing coagulation regulatory proteins hTBM and hEPCR and anti-apoptotic protein hCD47 as seen in Groups 3 and 4, compared to Group 2.

Additionally, coronary vasculopathy was found in xenografts of long-term survivors and possibly contributed to an acute myocardial infarction (MI) in one instance (B32988). Termed chronic allograft vasculopathy (CAV), this coronary disease is characterized by diffuse and concentric intimal hyperplasia.[33] It is known to be associated with intracoronary mural and occlusive thrombi and in some instances can lead to MI. CAV usually begins within the first year of transplant and is one of the leading causes of allograft failure.[34] Its pathogenesis is poorly understood, but likely has both immunologic and non-immunologic causes.[35–37] Randomized prospective studies have demonstrated that prophylactic treatment with statins reduces the rate of CAV and decreases mortality in heart transplantation patients.[38] This novel finding, combined with the knowledge gained from allotransplantation, suggests statin therapy should be added as a prophylaxis to mitigate chronic xenograft vasculopathy (CXV).

Results from Group 4 shared in this report are the first evidence of rejection free xenograft survival that provides a clinically translatable combination of GE pigs and target-specific immunosuppression without the need for additional adjuncts. It additionally provides insight and alternative solutions to address previously reported abnormal cardiac growth after OHTx transplantation. Our current immunosuppression protocol, summarized in table 2, is based largely on our previously demonstrated " Mohiuddin" regimen. Additions included two anti-inflammatory agents administered during the first 3 months post-transplantation (etanercept and tocilizumab) based on observations by us and others that these adjuncts may improve survival. However, peripheral cytokine analysis does not suggest any major differences in peripheral cytokine level between short and long-term survivors and those with anti-inflammatory genes

such as HO-1. Thus, the utility of these adjuncts have yet to be supported by data, but continue to be used nonetheless. The CD-40 co-stimulation blockade mAb used here (2C10R4) is not FDA approved for clinical use, but several clinical trials are in progress to test humanized forms of anti-CD40/CD40L mAb in transplant and autoimmune diseases (clinicaltrial.gov identifiers: NCT04711226, NCT04322149, NCT03781414, NCT03663335, NCT03905525, NCT03610516).

Conclusion:

In this study, we demonstrate clear pre-clinical efficacy to selective cardiac xenografts with carbohydrate antigen knockouts, GHR knockout and multiple human coagulation, complement and inflammatory regulatory proteins concomitantly with our proven immunosuppression protocol. Our results also demonstrate that removing Neu5Gc via CMAHKO may have detrimental effects in NHP models but should not be considered as evidence to not use CMAHKO xenografts in human xenotransplantation. Multigene xenografts may immediately reduce certain patients' morbidity and mortality as a bridge to allotransplantation, who would otherwise die waiting for a heart.

Competing Interest Statement:

David Ayares, Will Eyestone, Amy Dandro, Kasinath Kuravi, Lorri Sorrels and Todd Vaught are employed by Revivicor/United Therapeutics. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Funding of this study is generously provided by

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Materials and Methods:

Animals:

Specific Pathogen Free (SPF) *Papio albus* baboons (Southwest National Primate Center, San Antonio TX or MD Anderson Cancer Center, Bastrop TX) weighing 15-30 kg were screened to eliminate specific pathogens of interest and used as recipients. Weight-matched GE German Landrace pigs (Revivicor Inc., Blacksburg, VA) were used as xenograft donors. KO of up to three known carbohydrate antigens, Gal, SDa and Neu5Gc. and expression of multiple human genes, including CD46, TBM, EPCR, CD47 and HO-1. A subset of these GE pigs also had KO of GHR. Expression of transgenes was consistent and at high levels across all pigs by Western blot and immunohistochemistry quantification. A list of all the GE donor pig with different combination of multiple gene used in the study are shown in table 1. Euthanasia was performed for recipients with deteriorating condition of either cardiac or non-cardiac origin. Death was an endpoint in the event of unexpected rapid cardiac deterioration. All animals were used in compliance with guidelines provided by the Institutional Animal Care and Use Committee (IACUC) at the University of Maryland School of Medicine.

Genetic Engineering:

Cardiac xenografts were produced from genetically engineered swine generated by somatic cell nuclear transfer as previously described.[21] Transgene vectors expressing one, two, four or two plus four recombinant human proteins were transfected into cultured fetal porcine fetal fibroblasts (supplementary figure 10). Pigs in Group 1 harbored a multicopy minigene

expressing human CD46 and a multicopy vector expressing human thrombomodulin (TBM) driven by the endothelial-specific porcine TBM promoter.[39] Pigs in Group 2 contained a bi-cistronic vector containing a single copy each of human CD46 and human DAF, linked by a viral 2A sequence and driven by a single CAG promoter to ensure ubiquitous expression.[21] In addition to this bi-cistronic vector, Group 3 pigs harbored a tetra-cistronic vector in which TBM and EPCR were linked by a 2A sequence and driven by a single endothelial-specific promoter, plus human CD47 and HO1, linked by a 2A sequence and driven by CAG. Finally, Group 4 pigs contained a single tetra-cistron, similar to the Group 3 version except that the CAG-driven bi-cistron was designed to express human CD46 and CD47. The functional activity of each transgene was characterized in in vitro assays as previously reported. All vectors in Groups 1-4 were targeted to pre-selected landing pads in the genome, facilitated by crispr/Cas9 and homology-directed repair. The groups also contained various carbohydrate KOs as previously indicated. The dominant carbohydrate antigen galactose-α1,3-galactose (Gal) was knocked out by insertional mutagenesis of the Ggta1 gene that encodes α1,3 galactosyl transferase.[23] Two additional xenoantigens, Neu5Gc and SDa, were knocked out by crispr/Cas9-induced insertion and/or deletion mutations (indels) in genes encoding the enzymes that catalyze their synthesis, namely CMAH (cytidine monophosphate-N- neuraminic acid hydroxylase) and β4GalNT2 (β1,4-N-acetyl-galactosaminyltransferase), respectively. Lastly, growth hormone receptor (GHR) was knocked out with crispr/Cas9 induced indels to decrease the intrinsic growth of the heart.

Immunosuppression:

The immunosuppressive regimen for all recipient baboons included induction therapy and maintenance therapy, which is summarized in table 2. Induction included anti-thymocyte

globulin (Thymoglobulin; Genzyme, Cambridge, MA, USA; 5mg/kg on days -2 and -1), anti-CD20 antibody (Rituxan; Genetech, San Francisco, CA, USA; 19mg/kg on days , -7, 0, 7) for T and B cell suppression and anti-CD40 (clone 2C10R4) [15] (NHP Reagent Resource, Worcester, MA, USA; 50mg/kg on days -1, 0, 5, 9, 14 then per week) for blocking the CD40/CD154 co-stimulation pathway. Cobra venom factor (CVF; Quidel, San Diego, CA, USA; 50-100 Units; days -1, 0, and 1) or C1 Esterase Inhibitor (Berinert; CSL Behring, King of Prussia, PA, USA; 17.5un/kg; Days -1, 0 and 1) was used to inhibit the complement activation. Maintenance immunotherapy included Mycophenolate Mofetil (MMF; Genzyme, Cambridge, MA, USA; 20mg/kg BID, daily) and anti-CD40 monoclonal antibody (50mg/kg) weekly. All recipient baboons received continuous heparin infusion to keep the activated clotting time (ACT) level twice the baseline. Ganciclovir (Roche, Nutley, NJ, USA; 5 mg/kg/day) was administered daily Cytomegalovirus (CMV) prophylaxis. Other medications included Epogen (Amgen, Thousand Oaks, California; 200U/Kg) daily from day-7 to 7 and Cephazolin (Hospira, Lake Forest, IL, USA; 250mg) daily for 7 days were given. If there was any sign of abnormal xenograft function, and there was clinical suspicion of rejection, rescue therapy was initiated with intravenous bolus dose of Solu-Medrol (20mg/Kg for 3 days). Increased heparin dosage was also used to prevent thrombus formation, if needed and activated clotted time (ACT) was maintained twice the baseline. Tumor necrosis factor alpha inhibitor (Anti-TNFα) (Etanercept, Amgen Inc., Thousand Oaks, CA) and IL-6 inhibitors (Tocilizumab, Genentech, USA) were given for the first 3 months postoperatively.

Orthotopic Transplantation:

Preoperative transthoracic echocardiograms (TTEs) were conducted to ensure adequate cardiac function free of anatomic abnormalities deemed unsatisfactory for transplantation. Donor swine heart procurement was performed using 30cc/kg of blood cardioplegia with XVIVO© heart solution (XHS) for induction preservation (XVIVO© Perfusion, Gothenburg, Sweden). Cardiac preservation was performed using an XVIVO© Perfusion system with XHS cardioplegia at 8°C, maintaining a perfusion pressure of 20 mmHg in the aortic root and physiological pH (7.2-7.6). In two experiments, prior to the availability of XVIVO© technology, blood cardioplegia and storage preservation on ice was employed.

Life-supporting heart xenotransplantation was performed after placing the baboon recipient onto Aorto-bicaval cardiopulmonary bypass (CPB). Donor porcine xenografts were placed in the orthotopic position after native heart explantation, using a biatrial anastomosis technique.[40]

Postoperative Care:

A cardiac telemetry device (Data Sciences International (DSI) St. Paul, MN) was used to monitor hemodynamics, heart rhythm and temperature postoperatively. Baboons were extubated after hemodynamic stability was achieved on minimal ionotropic support and adequate cardiac function was demonstrated by transesophageal echocardiography (TEE). Drug administration, ionotropic support, and other supplemental drugs (if needed) were administered through a tunneled central line tethered through the recipient's cage. Critical care nurses and physicians provided ICU level monitoring and management for the first 48-72 hours postoperatively. As soon as deemed surgically appropriate, all baboons were placed on systemic heparin for an ACT

goal of twice the baseline level. Recipient blood pressure and heart rate thresholds were not used to guide the use of any afterload reduction or chronotropic controlling agents.

Methods for evaluating xenograft function:

Cardiac xenograft function was evaluated by telemetry (continuously; but weaned to intermittently every hour after 30 days) and transthoracic echocardiography (TTE). The telemetry device (DSI, St. Paul, MN USA) was implanted into the recipient baboon's chest as previously described to monitor the xenograft systolic and diastolic pressures, electrocardiogram (ECG), and the recipient's temperature in our HHTx model.[29,41] The telemetry device data was transmitted wirelessly to a receiver attached to the recipient's cage and analyzed on Ponemah® Software (DSI, St. Paul, MN USA). TTE's were obtained whenever the recipient was sedated, or as needed for clinical status change and need for evaluation.

Hematological and Biochemical Parameters of Recipients:

Complete blood count (CBC), which includes white blood cell (WBC) counts, hematocrit (HCT), red blood cells (RBC), hemoglobin, platelets, neutrophils and monocytes were analyzed by hemoanalyzer (Abaxis Vetscan HM5C, ZOETIS, Parsippany, NJ) and histochemistry (Abaxis Vetscan VS2, ZOETIS, Parsippany, NJ), was performed weekly for the first two months and then biweekly until the xenograft was explanted. Activated clotting time (ACT) and Troponin I levels were measured by iStat (Abbott Laboratories, Princeton, NJ, USA). IGF-1 levels were measured by Antech Diagnostics (Fountain Valley CA, USA).

610 Measurement of Non-Gal IgG and IgM Antibodies:

611

612 Non-gal antibodies (IgG and IgM) titer was measured in heat inactivated baboon serum by flow
613 cytometry using GTKO pAEC line (KO:15502).[42] pAEC lines was from miniature swine which
614 was kindly provided by Dr. Hendrik-Jan Schuurman, formerly of Immerge Biotherapeutics.
615 pAEC were isolated and cultured as previously described. Serum samples were collected from
616 baboon before and after transplant every 2-3 days for month and biweekly thereafter. Flow
617 cytometry was used to measure antibody binding (mean fluorescence intensity; MFI) with FITC
618 labeled anti human IgG (Cat # H10301) and IgM (Cat # H15101) antibodies (Invitrogen Corp,
619 Waltham, MA, USA) to porcine aortic endothelial cells on a Cytek Aurora (Fremont, CA)
620 Cytometer. The MFI of the cells were analyzed with Flowjo software (Flowjo LLC. Ashland,
621 OR, USA) for each test serum and compared with that produced by the controls.

622

623 Cytotoxicity Assay:

624 Sera from recipient baboons was tested in a CDC assay utilizing porcine aortic endothelial cells
625 (pAEC) with either donor or from the same genotype as donor (littermate). Heat inactivated
626 baboon serum samples diluted to 25% in culture media were applied to confluent PAEC
627 monolayers in 96 well plates for 30 min at 37°C. After incubation, serum dilutions were removed
628 and 7.5% rabbit complement (Bio-Rad, Hercules, California, USA) and IncuCyte® Cytotox Red
629 fluorophore reagent (Sartorius, Goettingen, Germany), diluted in culture media, was added to
630 each well. Cells were imaged and counted every 10-15m for 2h using a BioTek Cytation™5
631 reader (Winooski, Vermont) with a CY5 (628/685) filter set and high contrast bright field optics.
632 Percent cytotoxicity was calculated as the number of red fluorescent cells/total bright field cells
633 counted x100. Three replicate wells per serum sample were counted and the mean background

cytotoxicity from two untreated wells (complement only, no serum) for each cell line was subtracted from final results.

FACS analysis for T and B cells Phenotyping:

Immuno-staining was performed on PBMCs with fluorescence-conjugated antibodies. Anti-human CD3 (Cat#556611&#552852), CD4 (Cat#560628), CD5 (566193), CD8 (Cat#563822), CD16 (Cat#561725), CD20 (Cat#641405), CD24 (Cat#561646), CD25 (Cat#561405), CD28 (Cat#560683), CD45 (Cat#563530), CD95 (Cat#561635), CD127 (Cat#562437), CD138 (Cat#562098), IgD (Cat#563313), and IgM (Cat#562618) monoclonal antibodies from Pharmingen (BD Bioscience, San Francisco CA, USA) were used. Anti-human CD27 (Cat#302827) from Biolegend San Diego, CA, USA and anti CD19 (Cat#IM2470) from Beckman Coulter , Indianapolis, IN, USA was used. Anti-human CD21 (Cat#46-0219-42) and FoxP3 (Cat# 12-4777-42) was used from Invitrogen (Thermo Fisher Scientific) Waltham, MA. Antibodies (3 or 4 microliter per million cells) were used as recommended or suggested by the manufacturers. Samples were run on Cytek Aurora (Fremont, CA, USA). Flow Cytometry analysis was performed using Flow Jo Software (Flowjo LLC. Ashland, OR, USA).

Histological Evaluation of Biopsies and Explants of Xenografts:

Paraffin sections from multiple biopsies and sections of explanted xenografts were stained with hematoxylin and eosin for light microscopy. Sections were analyzed semi-quantitatively for the presence of hemorrhage, necrosis, thrombosis, and cellular infiltrates. Immunostaining for immunoglobulins (IgG (Cat# 760-2653), IgM (Cat# 760-2654) and complement (C3d (Cat#760-

4522) and C4d (Cat# 760-4436) Roche Tissue Diagnostics) on paraffin section were performed. All the specimens were examined by an independent pathologist. Aperio Digital Pathology Slide Scanner was used with 20x magnification, resulting in a field approximately 870 microns field size. Fields were chosen that had the least amount of necrosis to prevent secondary deposition of IgG and then examined for capillary staining for IgG, IgM and C4d. Intensity was measured for C4d staining as 0: negative or equivocal staining 1+: faint positive staining 2+: strong positive staining in accordance with the Society of Cardiovascular Pathology (SCVP), Association for European Cardiovascular Pathology (AECVP) and the International Society of Heart and Lung Transplantation (ISHLT). Additionally, specimens were graded for microthrombi on a scale of 0-4+, where 1+ = >0 capillaries stained; 2+ = >1-5 capillaries stained; 3+ = >5-10 capillaries stained, 4+ = >10 capillaries stained, per high power field.

Endomyocardial Biopsy:

Venous vascular access was obtained percutaneously through the right femoral vein. A 7 Fr sheath was then advanced over a wire with a balloon-tipped catheter from the venous sheath into the right ventricle using X-ray guidance. A bioptome was then advanced through the sheath and using telemetry monitoring to detect premature ventricular contractions. The bioptome was then opened advanced, closed, and withdrawn to remove a tissue piece 1mm cubed. For any given evaluation, 3-5 pieces are taken to be evaluated for pathological evidence of rejection. Venous closure was performed by manual pressure for 5-10 minutes.

Western blot of human transgene expression:

hTBM,hEPCR,hCD46, hCD47,hHO-1 and hDAF-1 protein detection in liver tissue lysates was carried out on automated capillary western blotting system Simple WES (Protein simple, San Jose, CA, USA). Liver tissue lysates were obtained using TPER buffer (Thermo Fisher Scientific, Waltham, MA, USA) with protease inhibitors (Thermo Fisher Scientific). Total protein concentrations were quantified using BCA kit (Thermo Fisher Scientific). 3µg of total protein was loaded into each well of 12-230 kDa WES separation module (Thermo Fisher Scientific). Anti-mouse detection module was used for mouse anti-human antibodies (hTBM, hEPCR, actin) and anti-rabbit detection was used for rabbit anti-human antibodies (hCD46,hDAF and hHO-1). Anti-sheep secondary antibodies were used for sheep anti-human CD47 antibody. All assays were run on manufacture's recommended default program.

Immunohistochemical detection of human transgene expression:

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissues. Samples were sectioned at 4µm, allowed to air-dry, deparaffinized in xylene and rehydrated through graded alcohols to water. Endogenous peroxidases were quenched using Dual Endogenous Enzyme Block (DEEB; Agilent Technologies, Santa Clara, CA, USA) for 10 minutes. After washing with TBS-Tween buffer (Sigma-Aldrich, St. Louis, MO, USA) sections were blocked with Serum Free Protein Block (Agilent Technologies) for 5 minutes. Sections were gently drained and 100µL of antibody was dispensed onto each slide and allowed to incubate at room temperature for 30 minutes. After incubation slides were rinsed in wash buffer then incubated in 100µL of EnVision+ Dual HRP secondary with 0.5% pig serum (Agilent Technologies) for 30

minutes. Slides were thoroughly rinsed in wash buffer. Antibody binding was then detected using five-minute incubation of 100µL of diaminobenzidine tetrahydrochloride (DAB, Agilent Technologies). Slides were rinsed in running tap water, counterstained with hematoxylin, dehydrated through graded alcohols and xylene, mounted on coverslips, and imaged. Positive staining was demonstrated by a deposition of brown pigment at the site of antibody binding.

Data Analysis:

All statistical analysis and graph tabulation were performed on GraphPad Prism 8 (San Diego, CA), including Kaplan Meyer curves and line graph plots.

Figures/Tables/Supplemental:

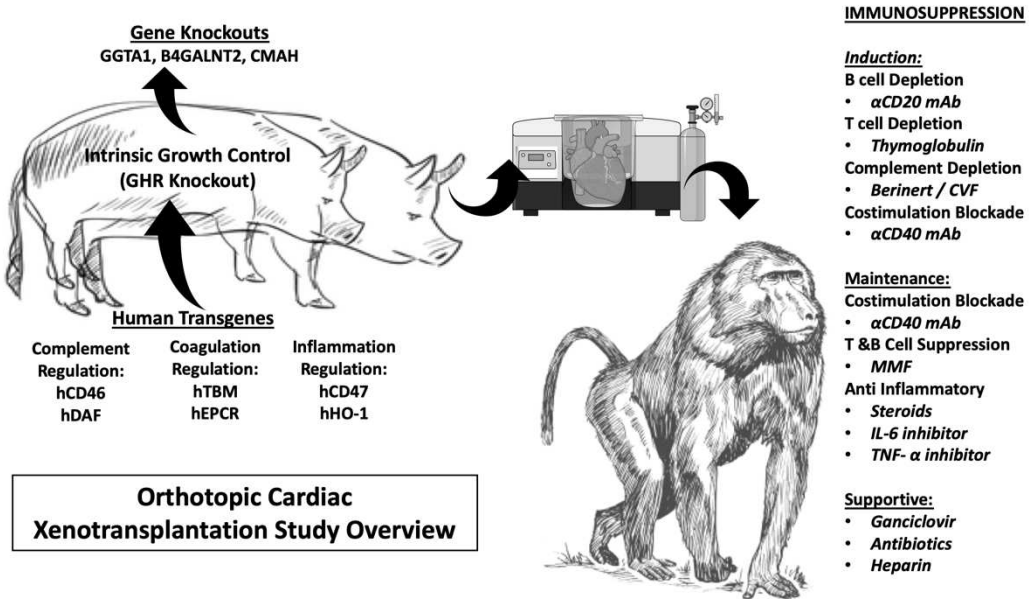


Figure 1: Study Overview. Pig-to-baboon xenotransplantation was performed with genetically modified pigs of various combinations. An anti-CD40 mAb-based regimen was used and

xenograft survival was measured. After euthanasia, the graft was explanted and examined. Multimodal analyses were performed on both the graft and the recipient.

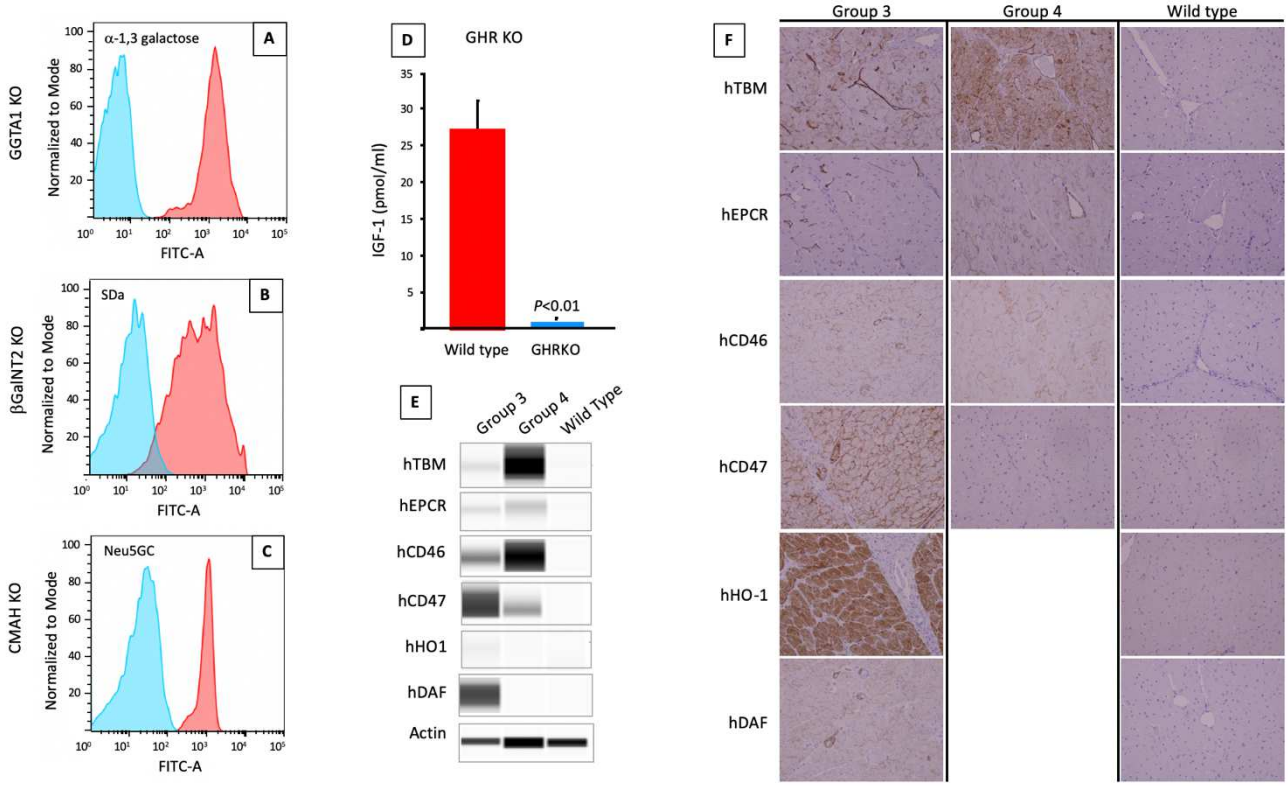


Figure 2: Xenograft phenotypes in long-term survivors. A, B, C: flow cytometry showing absence of a-1,3 galactose, SDa and Neu5Gc antigens after knockout of GGTA1, B4GalNT2, and CMAH, respectively in Group 2 pigs. Knockouts are shown in blue, wild types in red. CMAHKO (-/-) is shown in blue, whereas CMAHKO (+/-) has similar staining to wild type (not shown). D: Serum IGF-1 levels in GHR knockout donors in Group 4 (blue) vs. wild type pigs (red); E: western blot of human transgenes expression in tail biopsies of Group 3 and 4 donor pigs. F: IHC of explanted heart xenografts from Group 3 and 4 donors showing expression of human transgenes (x200).

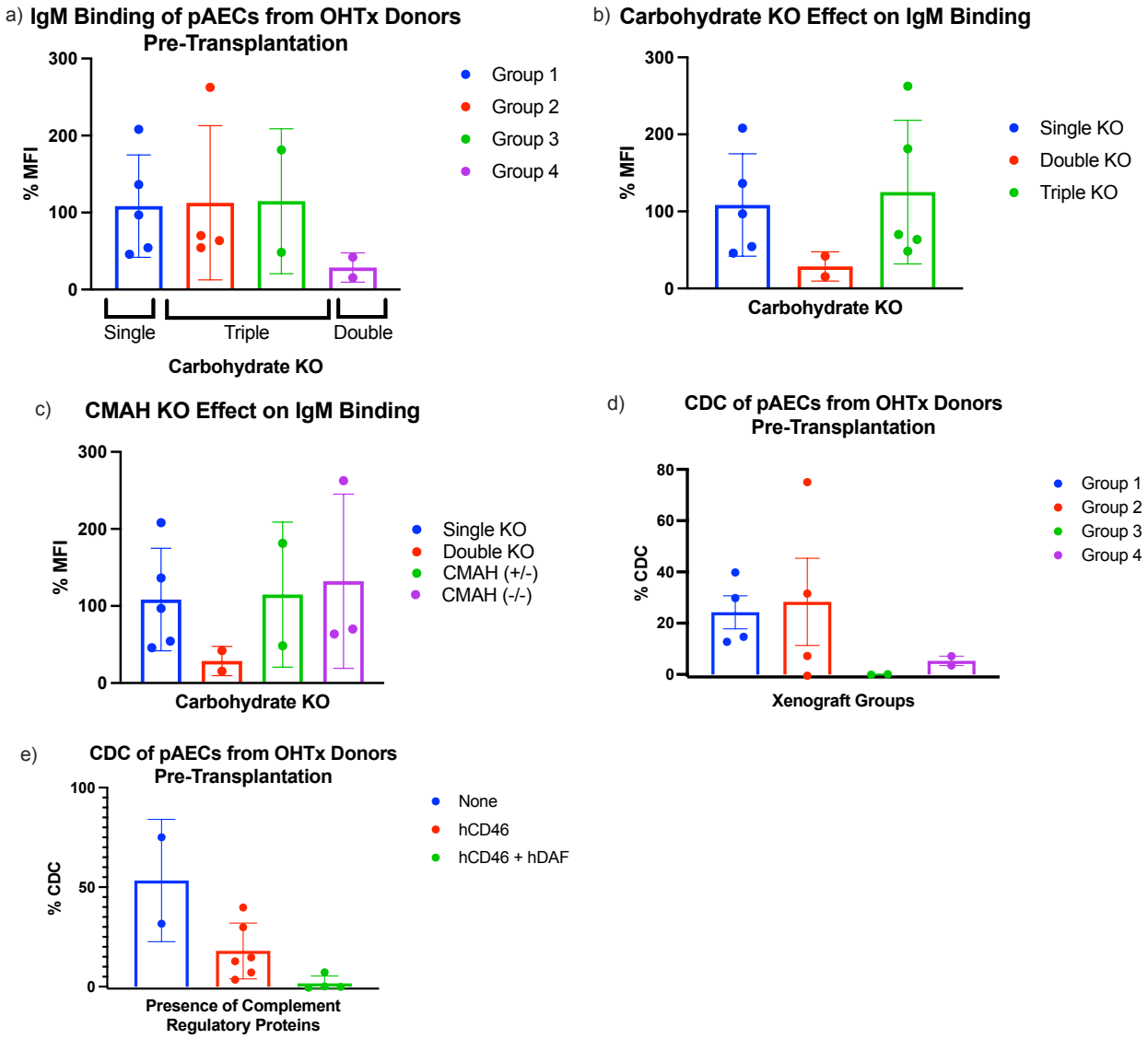


Figure 3: Characterization of multi-gene cardiac xenografts. a) IgM binding of pAECs from either xenograft donors or donor litter mates exposed to serum from Groups 1-4 recipients prior to OHTx. b) IgM binding from panel a, grouped by single, double or triple KO xenografts. c) IgM binding from a and b, grouped by CMAH (+/-) vs. (-/-). Single= GGTA1KO, double= GGTA1KO and B4GalNT2, triple=GGTA1KO, B4GalNT2 and CMAHKO. %MFI=MFI as a percent of control. CDC and IgM binding were performed as triplicates and presented here as an

average of triplicates. d) complement dependent cytotoxicity (CDC) measured on pig aortic endothelial cells (pAECs) from either xenograft donors or donor litter mates exposed to serum from Group 1-4 recipients prior to orthotopic transplantation (OHTx). e) CDC from panel d, grouped by complement regulatory proteins hCD46 and hDAF.

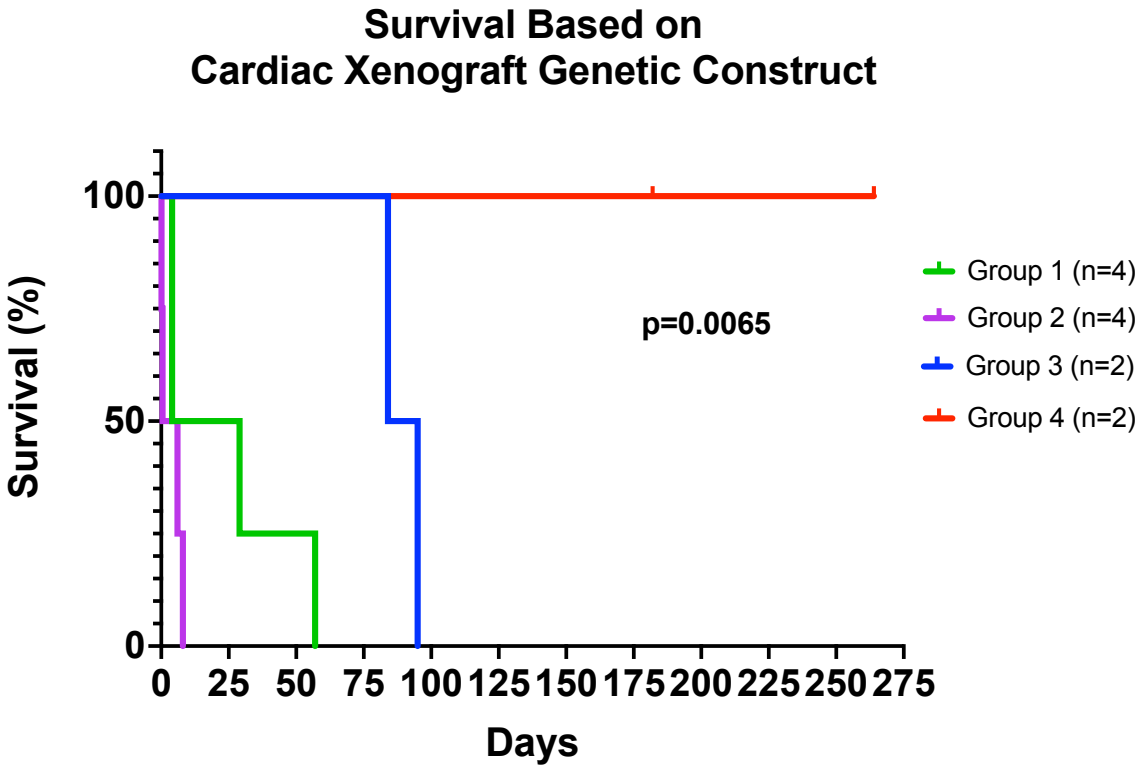
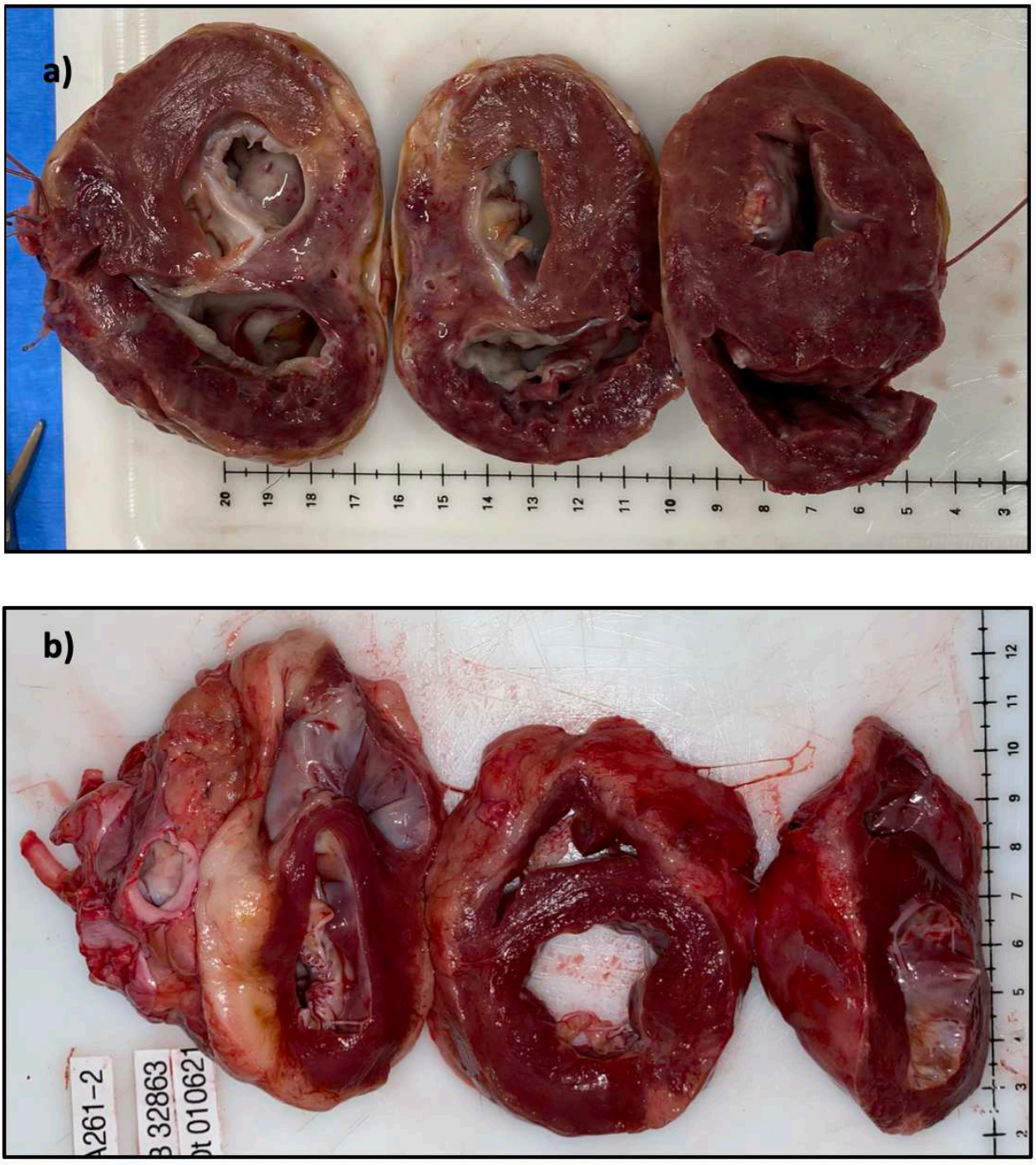


Figure 4: Recipient survival of Groups 1-4. Survival defined as time after transplantation before requiring euthanasia for deteriorating condition. Group 4 deaths censored for lack of evidence for immunologic rejection seen on histology, contributing to recipient deterioration. All other grafts contained histologic evidence of cardiac abnormalities contributing to deterioration requiring euthanasia. P=0.0065 by Log-rank (Mantel-cox) test, suggesting a significant difference in survival between Groups 1-4.

752



753 **Figure 5: GHRKO versus non-GHRKO xenografts.** a) non-GHRKO grafts (Group 3)

754 exhibited biventricular wall thickening. Here, B33121 survived 84 days prior to requiring

755 euthanasia for symptoms of diastolic heart failure. b) GHRKO graft (Group 4) exhibiting normal

histology without thickening at 182 days post-transplantation. This animal (B32863) was euthanized for weight loss as required by our institutional animal care committee, but was exhibiting excellent graft function.

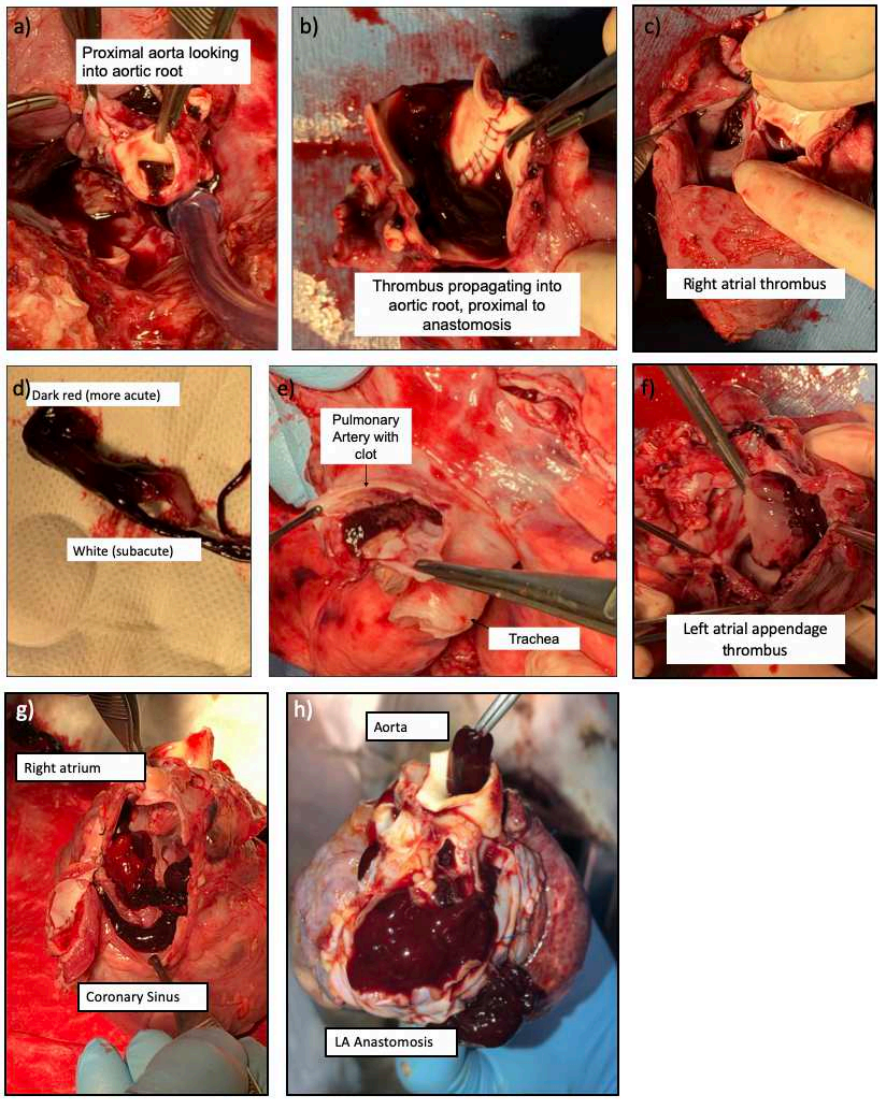
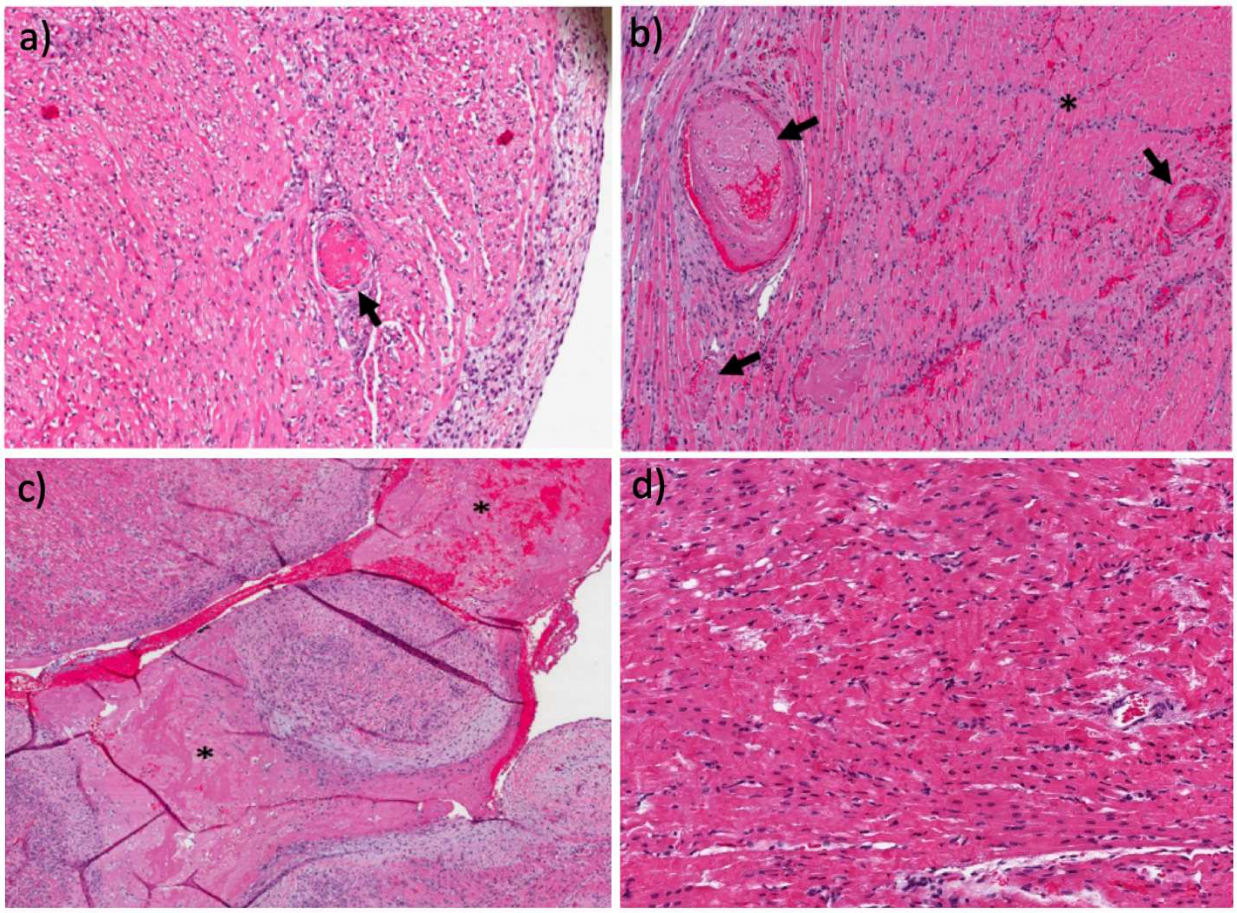


Figure 6a: Thrombotic complications in Group 2 (xenografts without thromboregulatory proteins). panels a-f, showing B33167's xenograft at explantation. Consists of propagating thrombus of the aortic root (a, b and d), left and right atrial thrombus (c and f) and pulmonary

764 artery (e). Pulmonary artery and left atrium appear to have acute and subacute components.
765 intracardiac thrombosis of B33156 within coronary sinus (g), aorta and pulmonary vasculature
766 (h).
767



768
769 **Figure 6b: Histologic findings on H&E in Group 2 (xenografts without thromboregulatory**
770 **proteins).** a) B33167 right ventricle, 10x magnification. Fibrin thrombus (arrow) in a
771 background of ischemic myocytes. b) B33156 apex, 10x magnification. Fibrin thrombi (arrows)
772 and a region of ischemic myocytes (asterisk). c) B33156 left ventricle, 10x magnification. Note
773 the intracardiac organizing thrombus (asterisk). d) B33060 right ventricle, 10x, note contraction
774 bands and hypereosinophilia, indicating an early necrotic process.
775

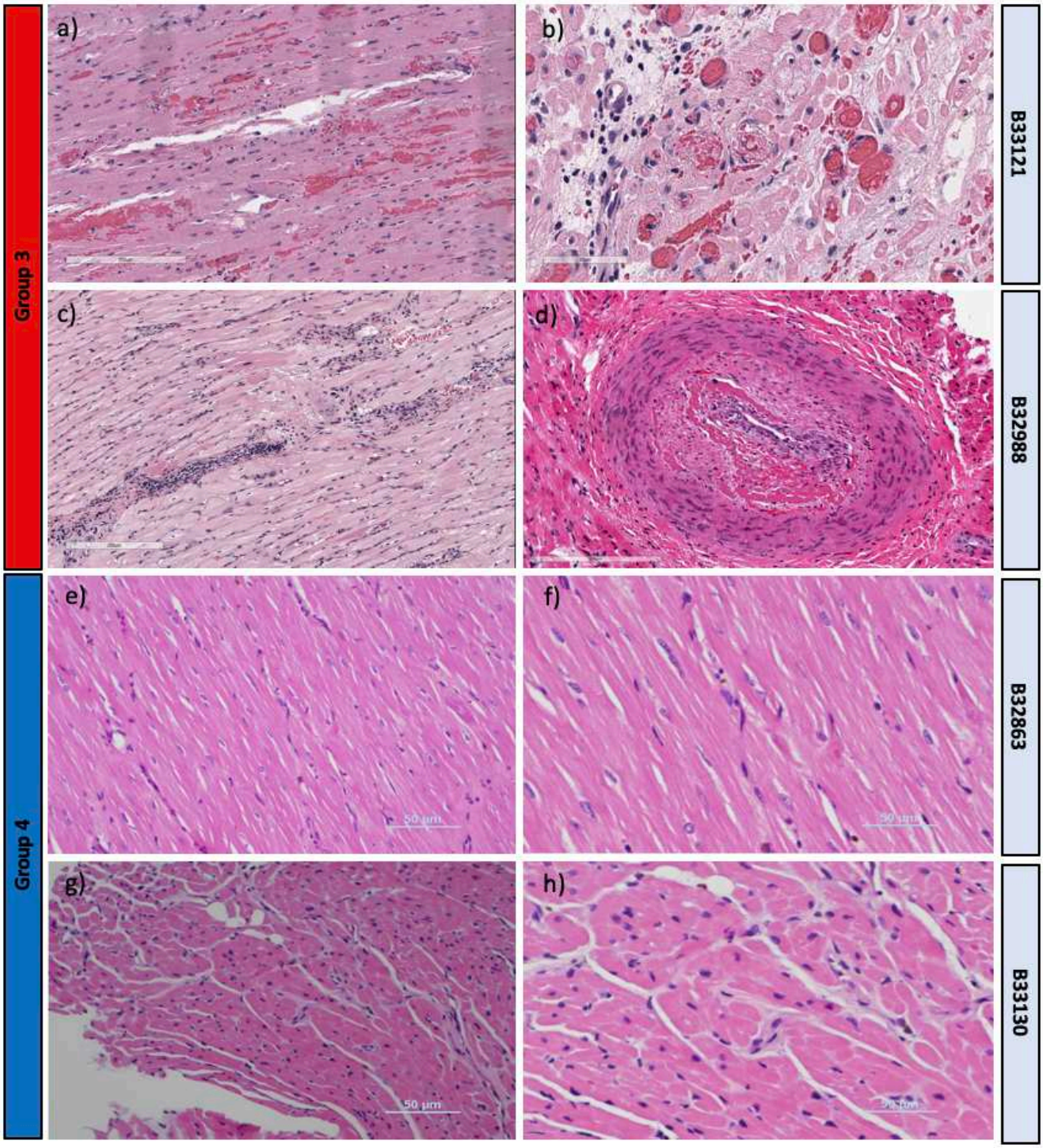


Figure 7: H&E in Long-term Survivors Between Groups 3 and 4. a) Group 3, B33121 LV (17X)- congestion, mild interstitial hemorrhage individual myofiber degeneration and necrosis, b) Group 3, B33121 RV (40X) veins with intravascular thrombosis c) Group 3, B32988 LV

(14X) interstitial mononuclear lymphoplasmacytic inflammation, scant perivascular hemorrhage, myodegeneration d) Group 3, B32988 RV (20X) organized thrombus in muscular artery, consistent with chronic xenograft vasculopathy e) Group 4, B32863 RV (20X) normal myocardium without evidence of rejection f) Group 4, B32863 RV (20X) normal myocardium without evidence of rejection g) Group 4, B33130 RV (20X), endomyocardial biopsy, normal myocardium without evidence of rejection h) Group 4, B33130 RV (40X), endomyocardial biopsy, normal myocardium without evidence of rejection

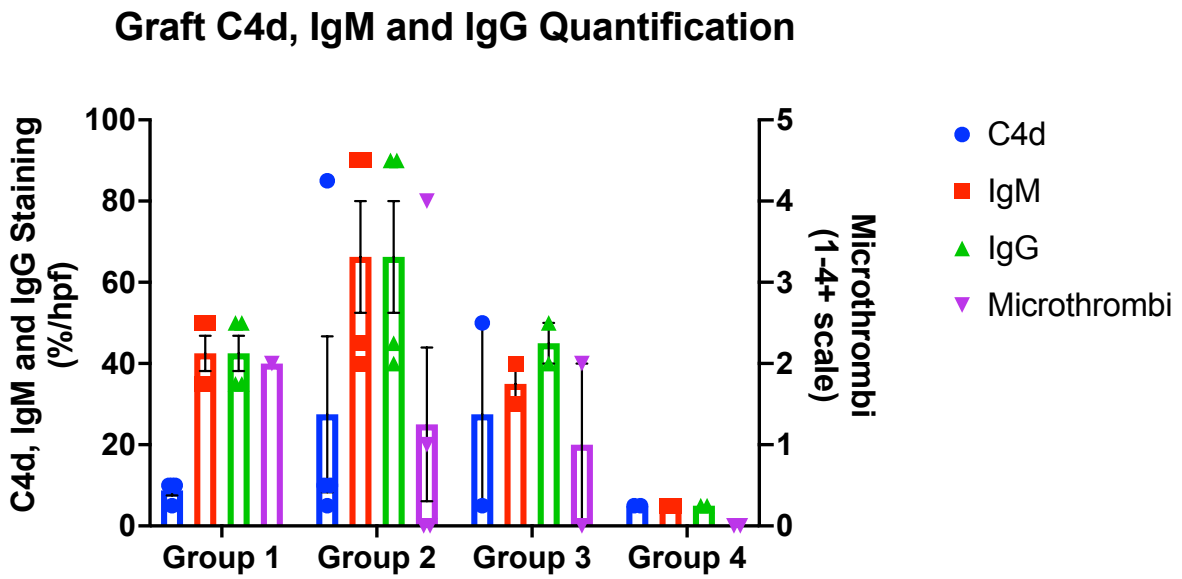


Figure 8: IHC quantification and microthrombi. Presented as averages within each group. High power field was with 20x magnification, resulting in approximately an 870 micron field. Microthrombi were graded on a scale of 0-4+, where 1+ = >0 capillaries stained; 2+ = >1-5 capillaries stained; 3+ = >5-10 capillaries stained, 4+ = >10 capillaries stained, per high power field.

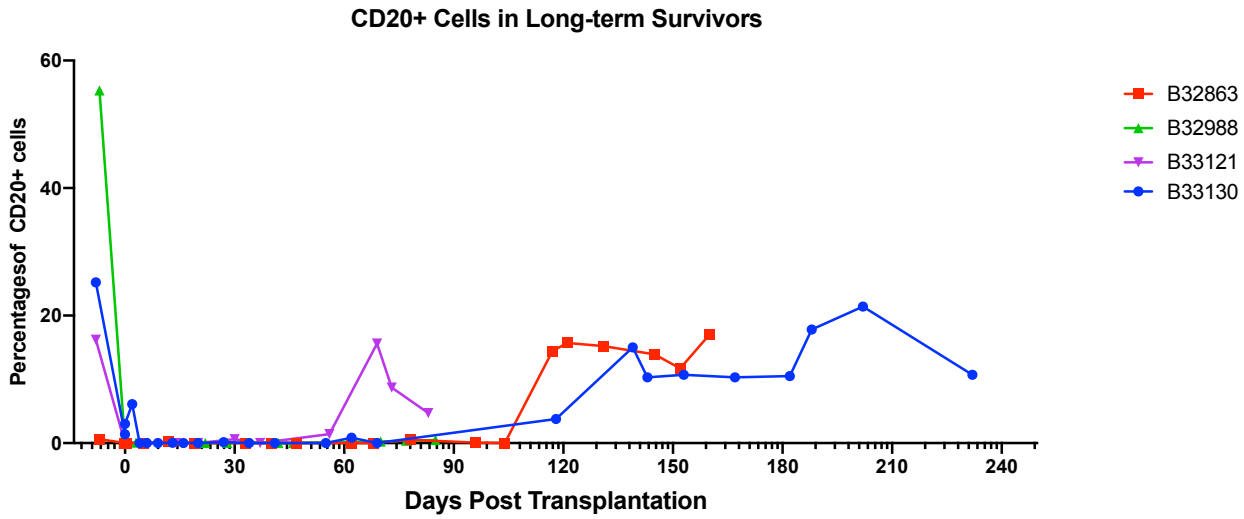


Figure 9: CD20+ cells in long-term survivors. B-cell depletion is adequate after induction therapy, followed by reconstitution by 60-90 days after transplantation. Percent CD20+ cells calculated as a proportion of total CD3+ cells. Group 3=B32988 and B33121; Group 4=B32863 and B33130.

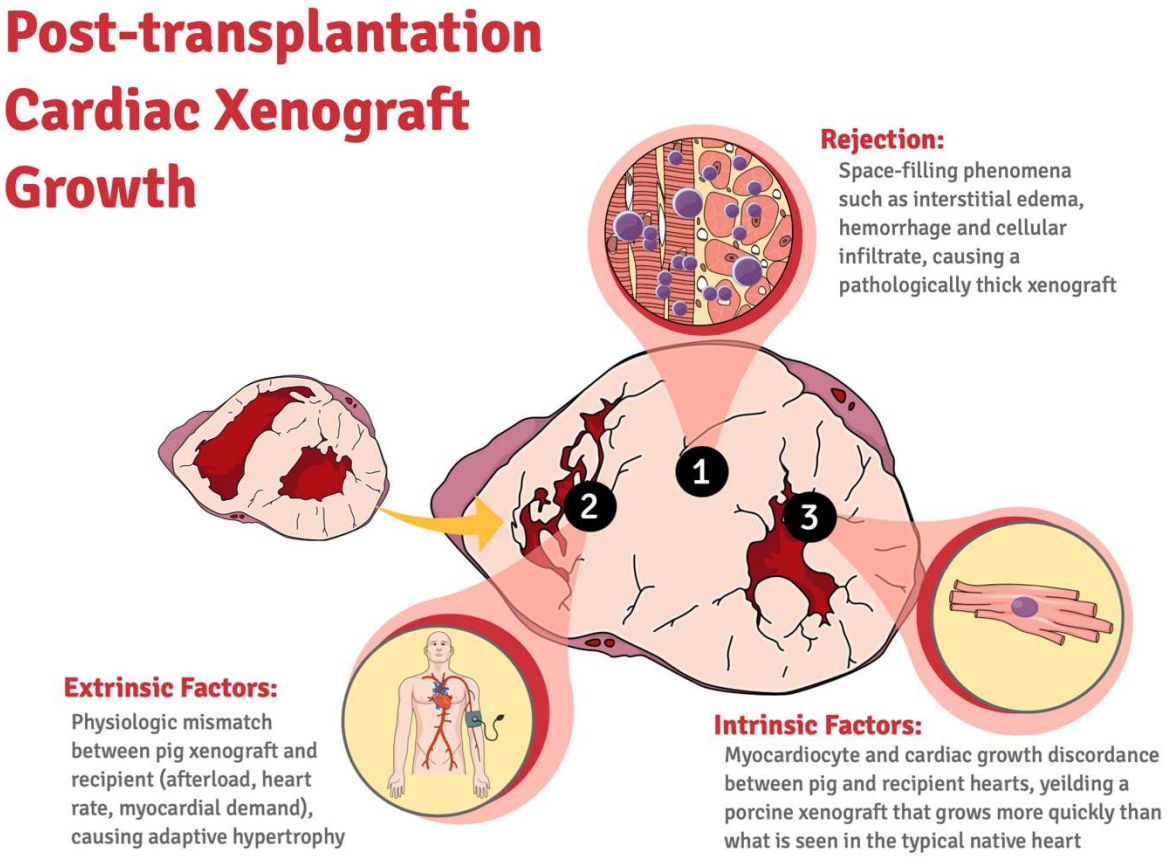


Figure 10: Potential mechanisms of post-transplantation cardiac growth in xenotransplantation. Post-transplantation cardiac xenograft growth is likely caused by both intrinsic and extrinsic factors, which includes rejection, intrinsic factors such as native xenograft growth. Other potential causes of growth include extrinsic factors such as physiologic mismatch leading to adaptive hypertrophy but was not observed in this study.

Table Legends:

Gene Knockout/Knockin #

Recipient ID #		Carbohydrate Enzyme Knockout			Other	Thromboregulatory		Complement Regulation		Anti-Inflammatory	
B32608	Group 1	GGTA1				TBM		CD46			
B32987		GGTA1				TBM		CD46			
B33422		GGTA1				TBM		CD46			
B32628		GGTA1				TBM		CD46			
B33167	Group 2	GGTA1						CD46	DAF		
B32638		GGTA1	β4GalNT2	CMAH (-/-)				CD46	DAF		
B33156		GGTA1	β4GalNT2	CMAH (-/-)							
B33060		GGTA1	β4GalNT2	CMAH (-/-)							
B33121	Group 3	GGTA1	β4GalNT2	CMAH (+/-)		TBM	EPCR	CD46	DAF	CD47	HO1
B32988		GGTA1	β4GalNT2	CMAH (+/-)		TBM	EPCR	CD46	DAF	CD47	HO1
B33130	Group 4	GGTA1	β4GalNT2		GHRKO	TBM	EPCR	CD46		CD47	
B32863		GGTA1	β4GalNT2		GHRKO	TBM	EPCR	CD46		CD47	

Table 1: Groups by Knockout and Human Transgene Expression. Human transgenes are categorized by thromboregulatory, complement regulation and anti-inflammatory proteins. GGTA1= α1,3-galactosyltransferase, β4GalNT2=β1,4-N-acetylgalactosyltransferase, CMAH= CMP-N-acetylneuraminic acid hydroxylase, TBM=thrombomodulin, EPCR=endothelial protein C receptor, DAF=decay accelerating factor, HO1=hemeoxygenase, GHRKO=growth hormone receptor knockout. CMAH (-/-)=knockout homozygous at this locus, CMAH (+/-)=knockout heterozygous at this locus.

Induction						
Induction						
	Agent	**Dose**	**Timing**	**Route**	**Pre-Treatment**	**Purpose**
	Anti-CD20	19 mg/kg	Day -7, 0, 7	IV infusion	Solu-Medrol, Benadryl, H2 blocker	To deplete B-cells
	ATG	5 mg/kg	Day -2, -1	IV infusion	Solu-Medrol, Benadryl, H2 blocker	To reduce number of T-cells
	Anti-CD40 (clone 2C10R4) CVF or Berinert	50 mg/kg 50-100 U/kg	Day -1 and 0 Day -1, 0 and 1	Slow IV infusion	None None	Co-stimulation blockade. Suppression of both B- and T-cell response. To inhibit complement activity
	Tocilizumab	8 mg/Kg	Day 0	IV	None	Anti-Inflammatory
	Etanercept	0.7 mg/Kg	Day 0	SC	None	Anti-inflammatory
Maintenance						
	Anti-CD40 (clone 2C10R4)	50 mg/kg	Days 3,5,9,14 then weekly	Slow IV infusion	None	Co-stimulation blockade. Suppression of both B- and T-cell response.
	MMF	20 mg/kg/2hr	BID, daily	IV infusion	None	B and T cell suppression
	Tocilizumab	8 mg/Kg	Weekly until day 90	IV	None	Anti-Inflammatory
	Etanercept	0.7 mg/Kg	Weekly until day 60	SC	None	Anti-inflammatory
	Solu-Medrol	2 mg/kg	BID tapered off in 7 weeks	IV	None	Suppress inflammation
	Aspirin	40 mg	Daily	Oral	None	Prevent platelet aggregation
	Heparin	50-1,000 U/hr	Continuous	IV infusion	None	Maintain ACT 2X normal and prevent inflammation
Supportive						

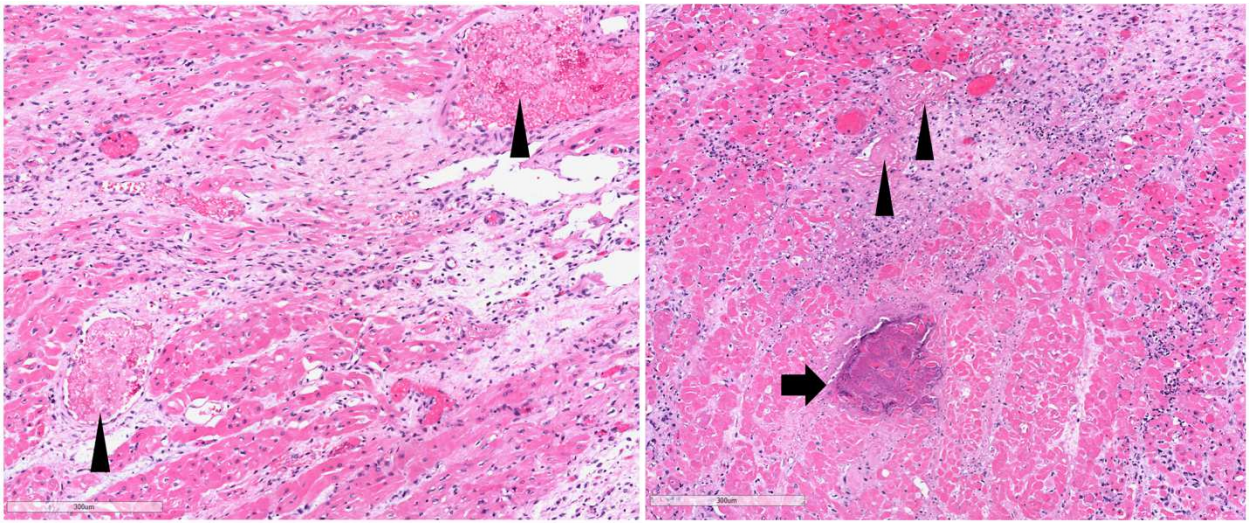
	Ganciclovir	5 mg/kg/day	Daily	IV infusion	None	For CMV prophylaxis
	Cefazolin	250 mg	TID daily for 7 days and whenever needed	IV	None	Infection prophylaxis and treatment
	Epogen	200 U/kg	Day -7 to 7 then weekly	IM or IV	None	To increase hematocrit

Table 2: Immunosuppression Regimen. 125 mg Solu-Medrol is given before Rituxan, ATG at 20mg/kg. Solu-Medrol 20mg/Kg/daily is given for 3 days at any concern for rejection. IV=intraveneous, SC=subcutaneous, BID=twice daily, TID=three times daily

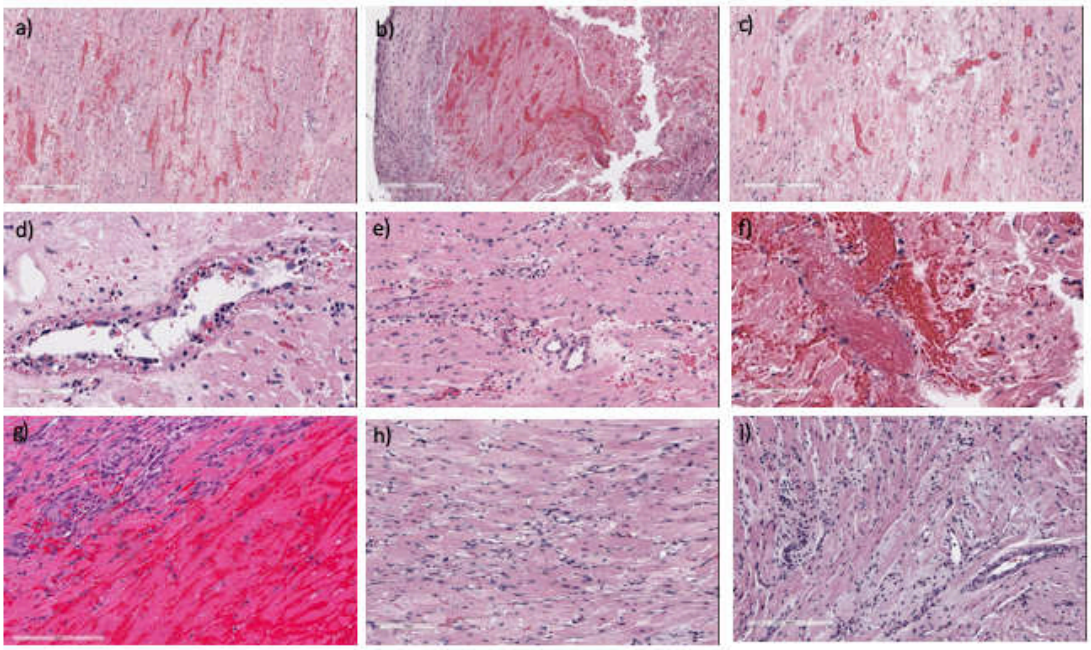
Supplementary Legends:

Group	Recipient ID	Donor Weight (kg)	Recipient Weight (kg)	Cross Clamp Time (minutes)	Total Ischemia Time (minutes)	CPB Time (minutes)	Preservation	Survival
Group 1	B32608	12	13	142	-	152	BC	6 hours
	B32987	20	17	50	55	92	BC	4 days
	B33422	21	21	49	95	102	BC	29 days
	B32628	21.1	22	53	156	98	XHS	57 days
Group 2	B33167	18	22	63	210	132	XHS	6 days
	B32638	16.8	17.3	47	66	126	XHS	12 hours
	B33156	24	23	31	159	91	XHS	8 days
	B33060	24.7	24.7	40	108	227	XHS	n/a
Group 3	B33121	18	18.6	-	208	117	XHS	84 days
	B32988	28	23	66	173	-	XHS	95 days
Group 4	B33130	31.5	27.4	82	252	129	XHS	264 days
	B32863	33.6	29	68	187	116	XHS	182 days*

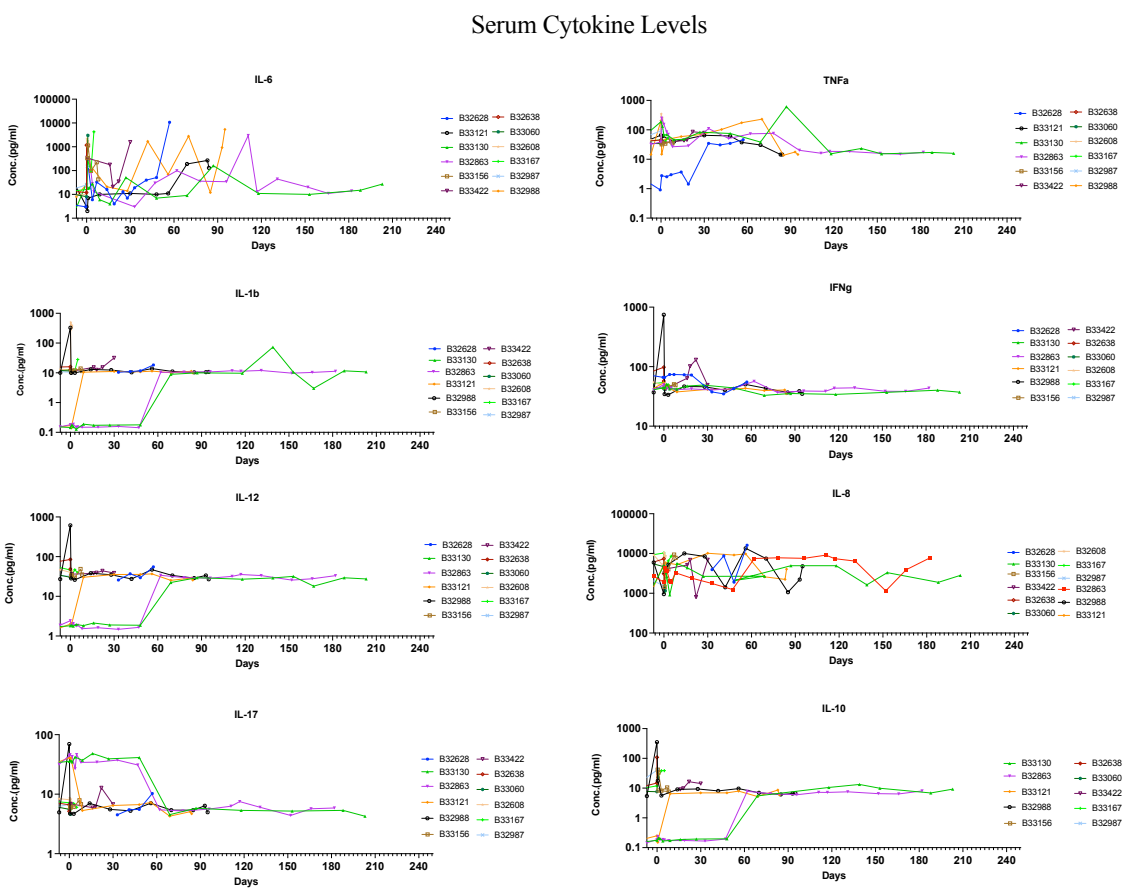
Supplementary Table 1: Overview. CPB=cardiopulmonary bypass time, BC=blood cardioplegia, XHS=XVIVO heart solution with NICP, survival with ">", indicates survival is ongoing. *=elective euthanasia



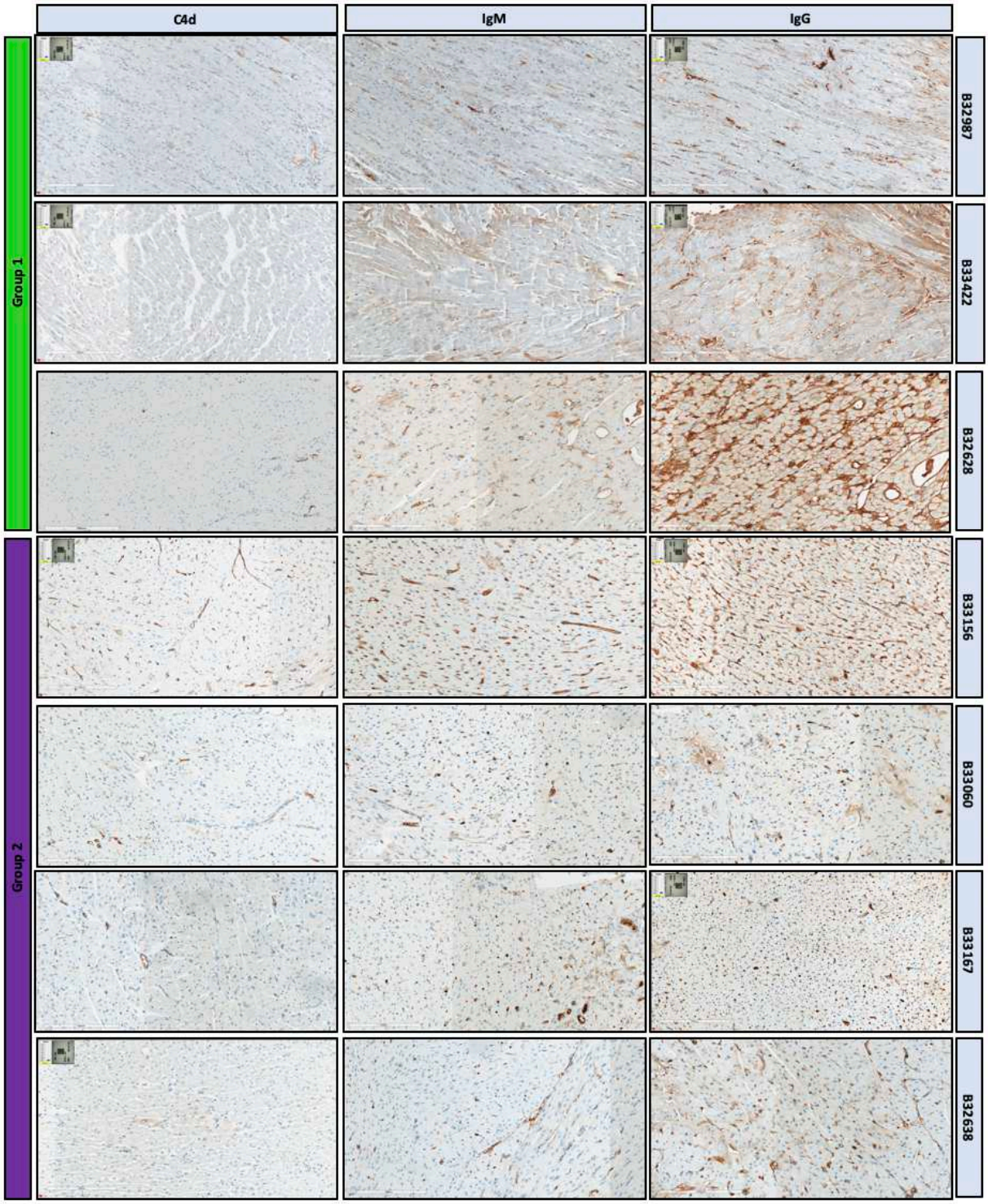
Supplementary Figure 1: H&E suggestive of AMR in B32628. Left panel-Right ventricle, 10x magnification, H&E stain: two large vessels with fibrin thrombi (arrowheads), Right panel-Right ventricle, 10x magnification, H&E stain: fibrin thrombi (arrowheads) and necrotic myocytes with infiltrating neutrophils and focal calcification (arrow).



Supplemental Figure 2: Space occupying lesions in grafts with rejection or inflammation. a) Septum (10X) interstitial hemorrhage, b) Septum (10X) myonecrosis subepicardial with hemorrhage epidcardial fibrosis, c) Septum (10X) myonecrosis, congestion, microthrombi, d) RV (40X) vasculitis with fibrinoid degeneration-necrosis e) LV (20X) mild interstitial hemorrhage, edema, scant mononuclear perivascular inflammation f) Septum (40X) fibrin microthrombi, hemorrhage and surrounding myonecrosis and degeneration g) septum (20X)-infarct about 7 days old i) RV (10X) myocardial degeneration, mild fibrosis, mild mononuclear inflammation and mild interstitial hemorrhage j) LV (20X) myofiber degeneration, atrophy, and fibrosis. B33121: panels a-f, B32988: panels g-i.

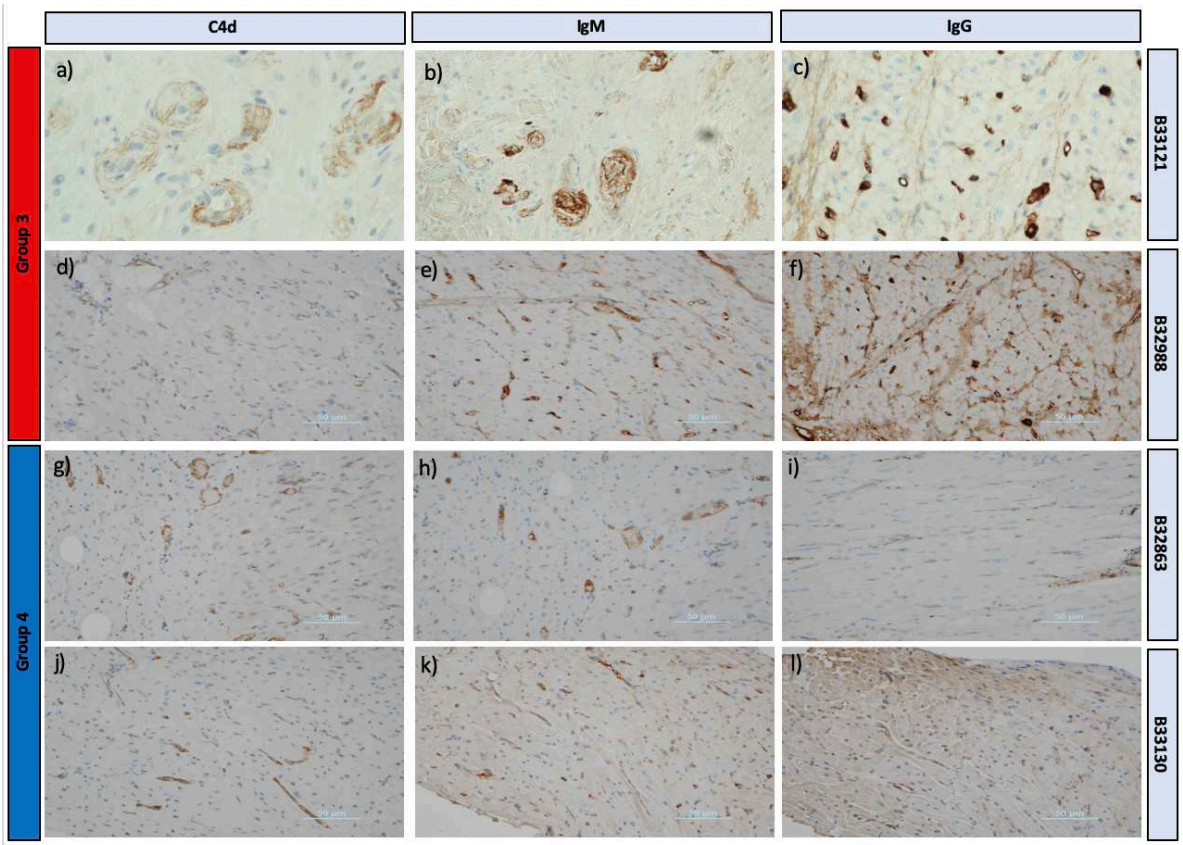


Supplementary Figure 3: Peripheral cytokine analysis in recipients of GE xenografts. Longitudinal peripheral cytokine analysis reveals no major differences between recipients of different GE xenografts. Toculizimab (IL-6 inhibitor) and Etanercept (TNF-alpha inhibitor) were stopped 120 days after transplantation.



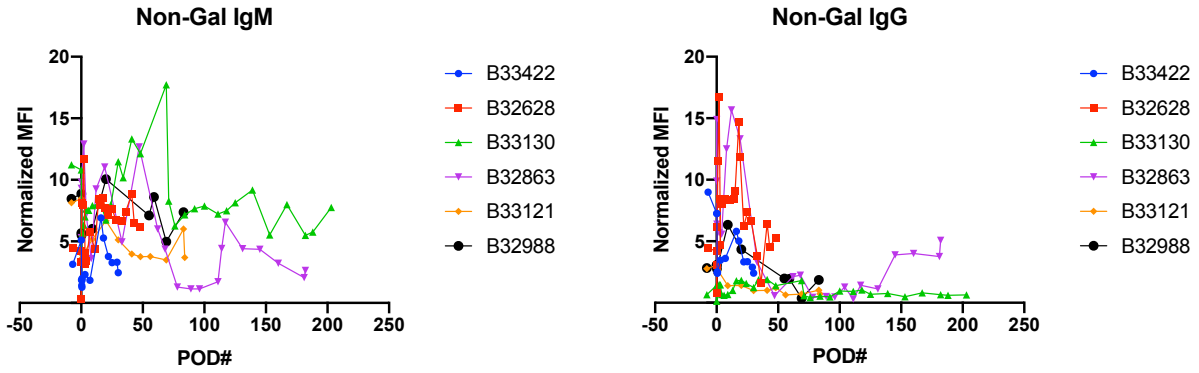
Supplemental Figure 4a: IHC for C4d, IgM and IgG in Xenografts in Groups 1 and 2, RV (20X). demonstrating increased C4d staining in Groups 2, compared to Group 1. The addition of

complement regulatory proteins (hCD46 and hDAF) in Group 2 reduces C4d staining similar to Group 1. IgM/IgG was more variable, with staining in Group 1 from 30-50% of capillaries and Group 2 between 40-90%.

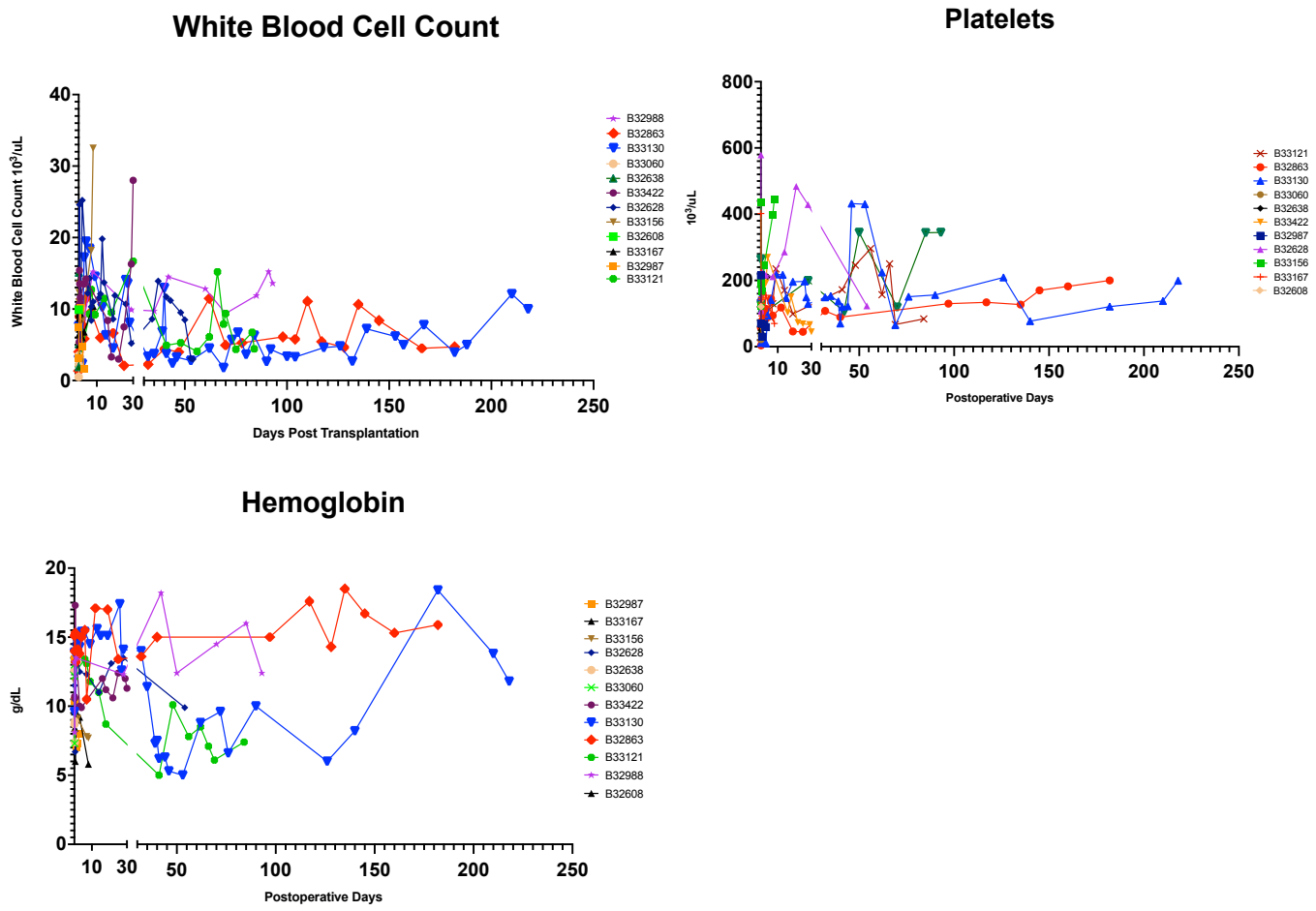


Supplementary Figure 4b: IHC for C4d, IgM and IgG in Xenografts between Groups 3 and Group 4, RV (20X). a) staining of C4d complement degradation product in <5% of capillaries, b) Strong IgM staining of 30% of capillaries, c) Strong IgG staining of 50% of capillaries d) C4d complement degradation product staining mild (1+: faint positive staining) in 50% of capillaries d) IgM staining of 40% of capillaries, f) IgG staining of 40% of capillaries g) RV (20X) C4d complement degradation product in <5% of capillaries h) RV (20X) IgM staining of <5% of capillaries, i) RV (20X) IgG staining of <5% of capillaries, IgM/IgG only showing non-specific,

non-endothelial serum j) RV C4d staining <5 % of capillaries k) IgM staining <5 % of capillaries l) IgG staining <5 % of capillaries, note: IgM/IgG in panels h, I, k, l showing largely non-specific, non-endothelial serum staining



Supplementary Figure 5: anti-pig antibody levels as measured by flow cytometry in long-term survivors of cardiac xenografts. MFI normalized to positive control. POD=postoperative day.



876 **Supplementary Figure 6: Complete Blood Count (CBC) over time for all recipients.**

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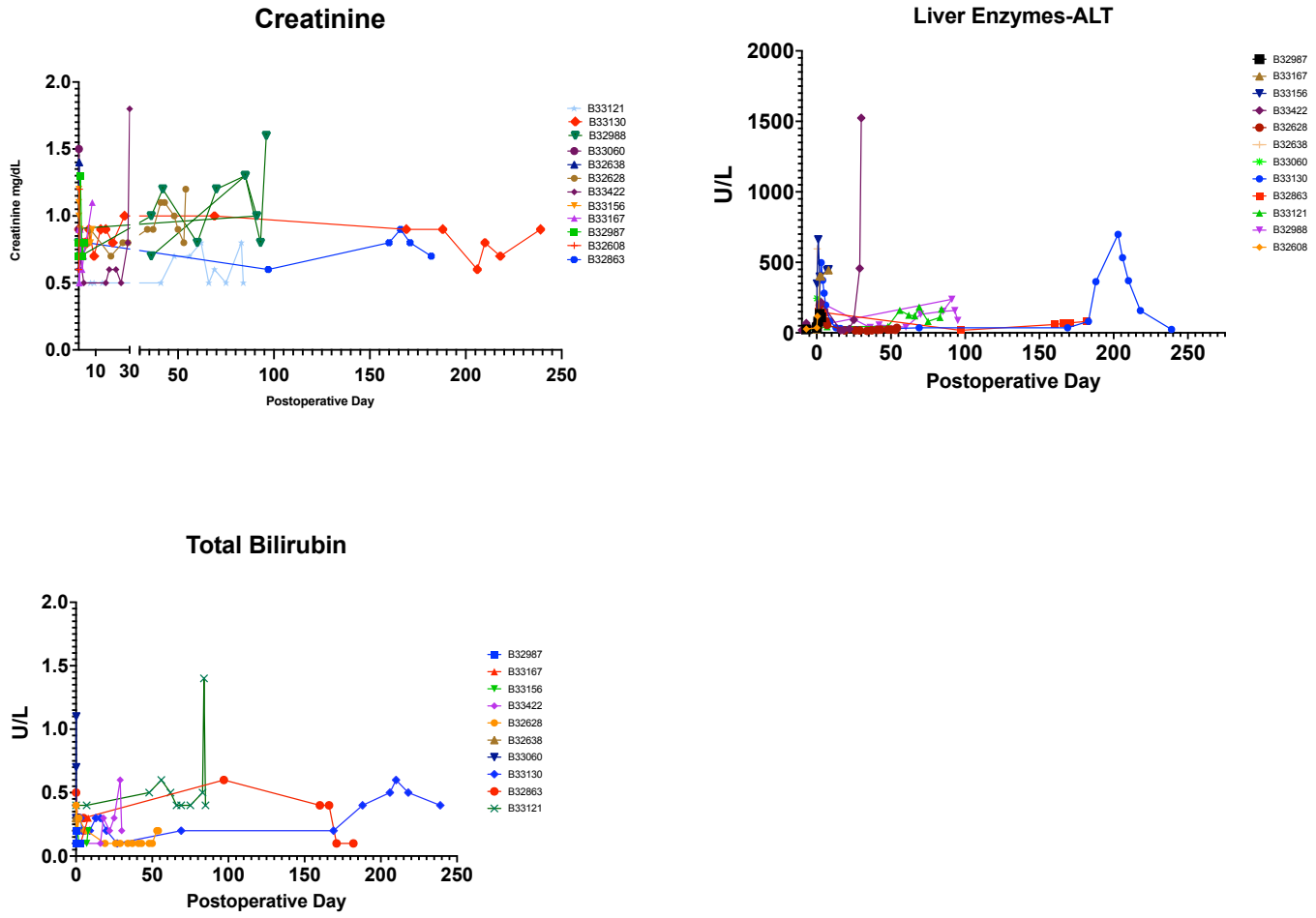

882 **Supplementary Figure 7: Metabolic Panel (CMP) over time for all recipients.**

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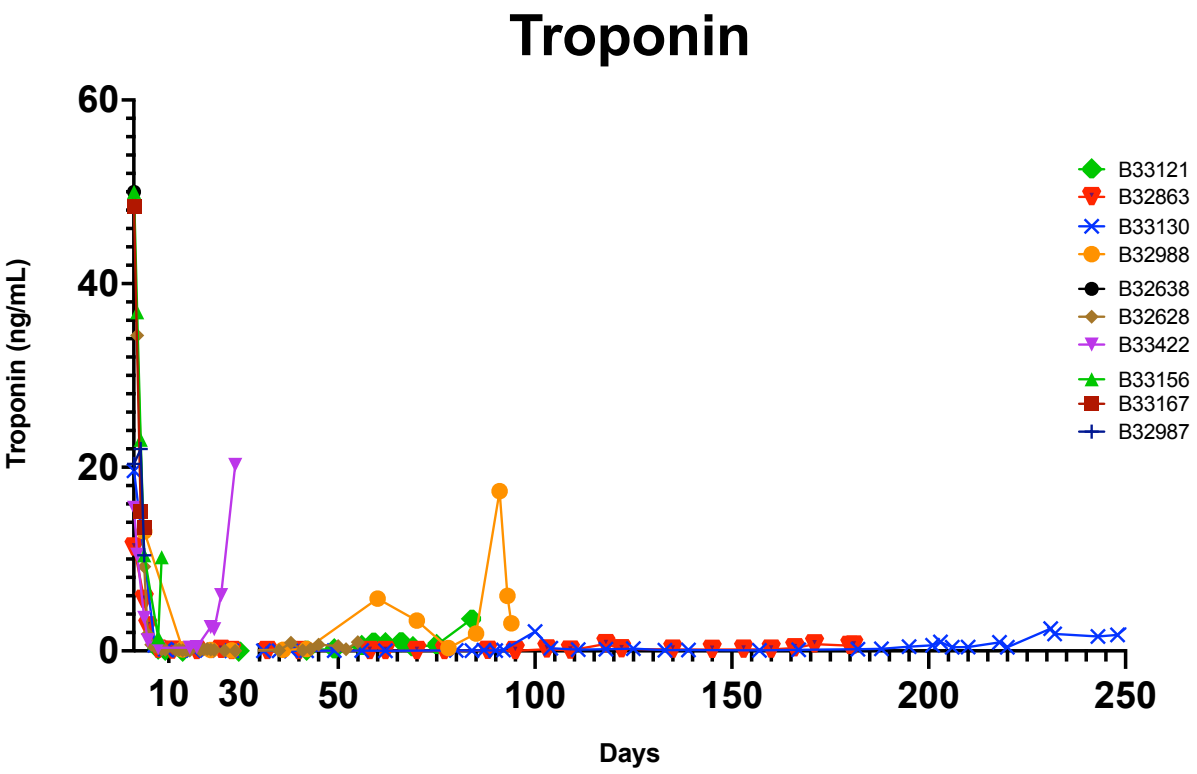

885 **Supplementary Figure 8: Troponin I over time post-transplantation.**

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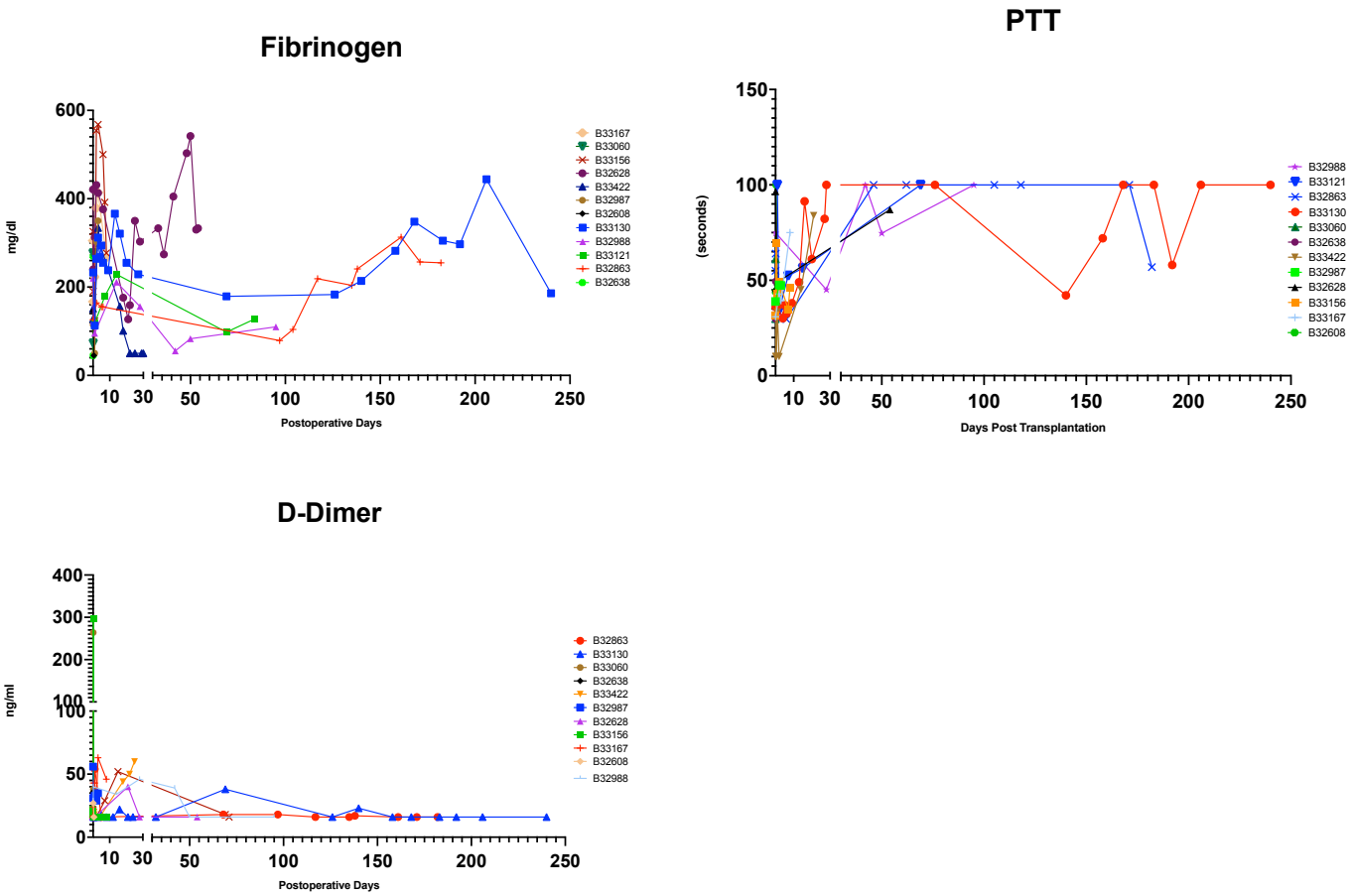

888 **Supplementary Figure 9: Coagulation Profile over time for all recipients. PTT=partial**
889 **thromboplastin time.**

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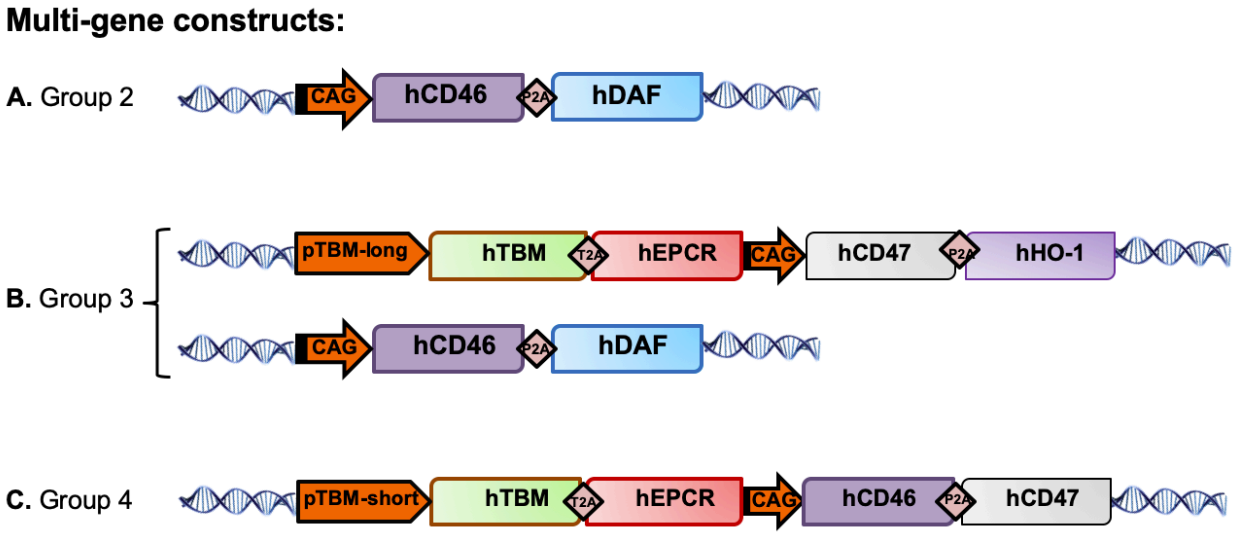

Supplementary Figure 10: Transgene expression vectors in multi-gene pigs. A) CAG-driven bi-cistronic vector used in Group 2, expressing human CD46 and DAF via a viral 2A sequence; B) Two vectors used in Group 3: a tetra-cistronic vector consisting of a bi-cistron expressing human TBM and EPCR, both driven by the porcine TBM promoter via a 2A sequence, coupled to a second, CAG-driven bi-cistron expressing human CD47 and HO1; Group 3 also contained the same bi-cistronic vector used in Group 2; C) a single tetra-cistronic vector was used in Group 4, consisting of a human TBM and EPCR driven by the porcine TBM promoter, coupled to a CAG-driven bi-cistron expressing human CD46 and CD47.

Figures

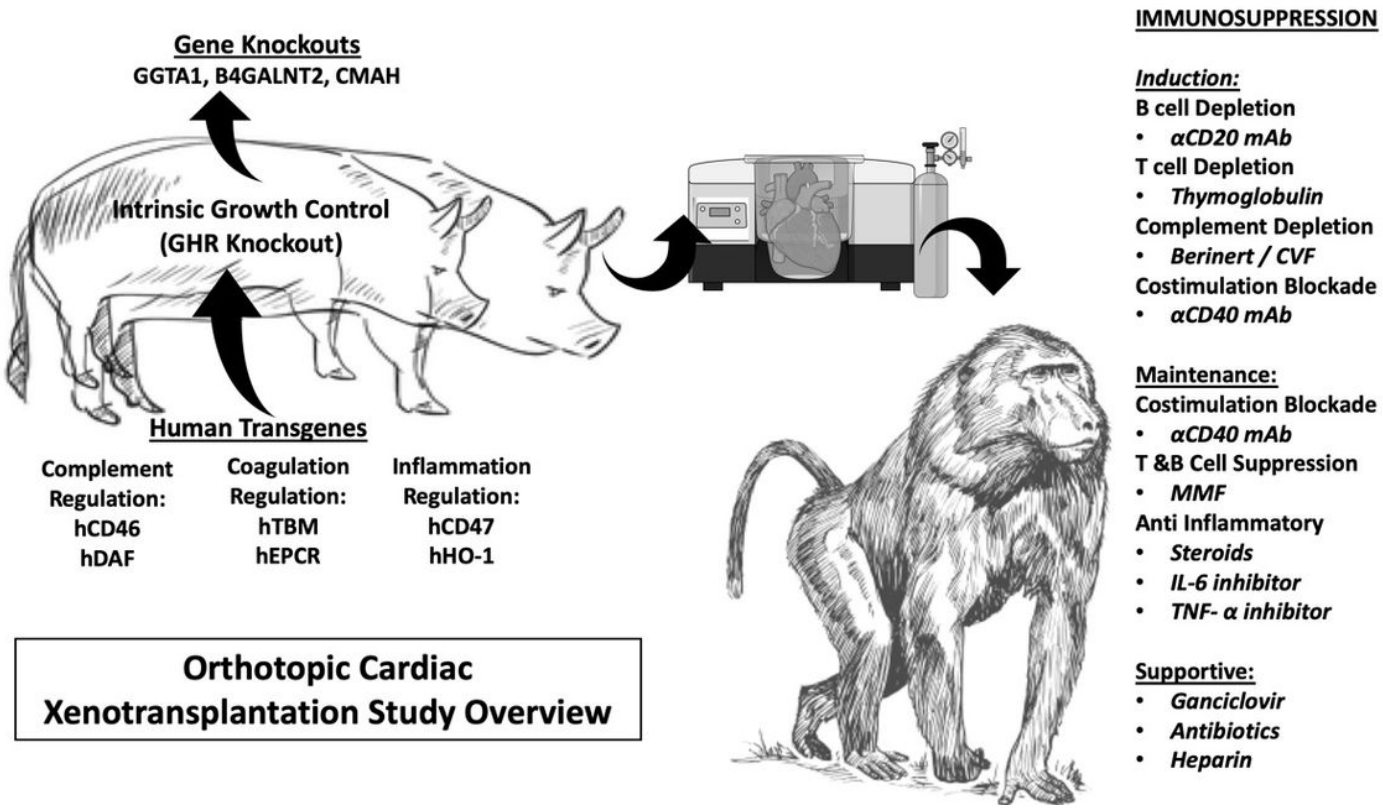

Figure 1

Study Overview. Pig-to-baboon xenotransplantation was performed with genetically modified pigs of various combinations. An anti-CD40 mAb-based regimen was used and xenograft survival was measured. After euthanasia, the graft was explanted and examined. Multimodal analyses were performed on both the graft and the recipient.

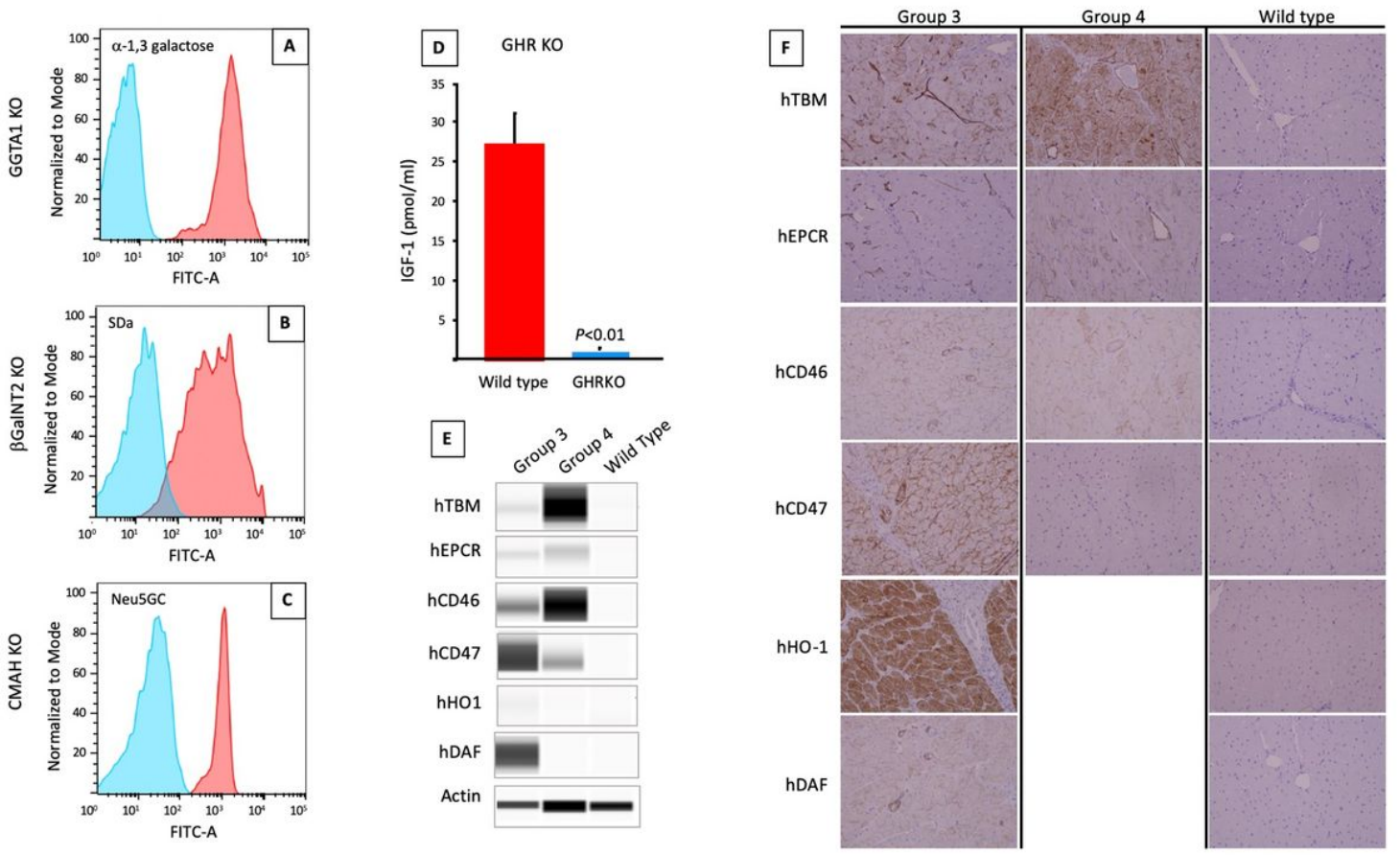

Figure 2

Xenograft phenotypes in long-term survivors. A, B, C: flow cytometry showing absence of a-1,3 galactose, SDa and Neu5Gc antigens after knockout of GGTA1, B4GalNT2, and CMAH, respectively in Group 2 pigs. Knockouts are shown in blue, wild types in red. CMAHKO (-/-) is shown in blue, whereas CMAHKO (+/-) has similar staining to wild type (not shown). D: Serum IGF-1 levels in GHR knockout donors in Group 4 (blue) vs. wild type pigs (red); E: western blot of human transgenes expression in tail biopsies of Group 3 and 4 donor pigs. F: IHC of explanted heart xenografts from Group 3 and 4 donors showing expression of human transgenes (x200).

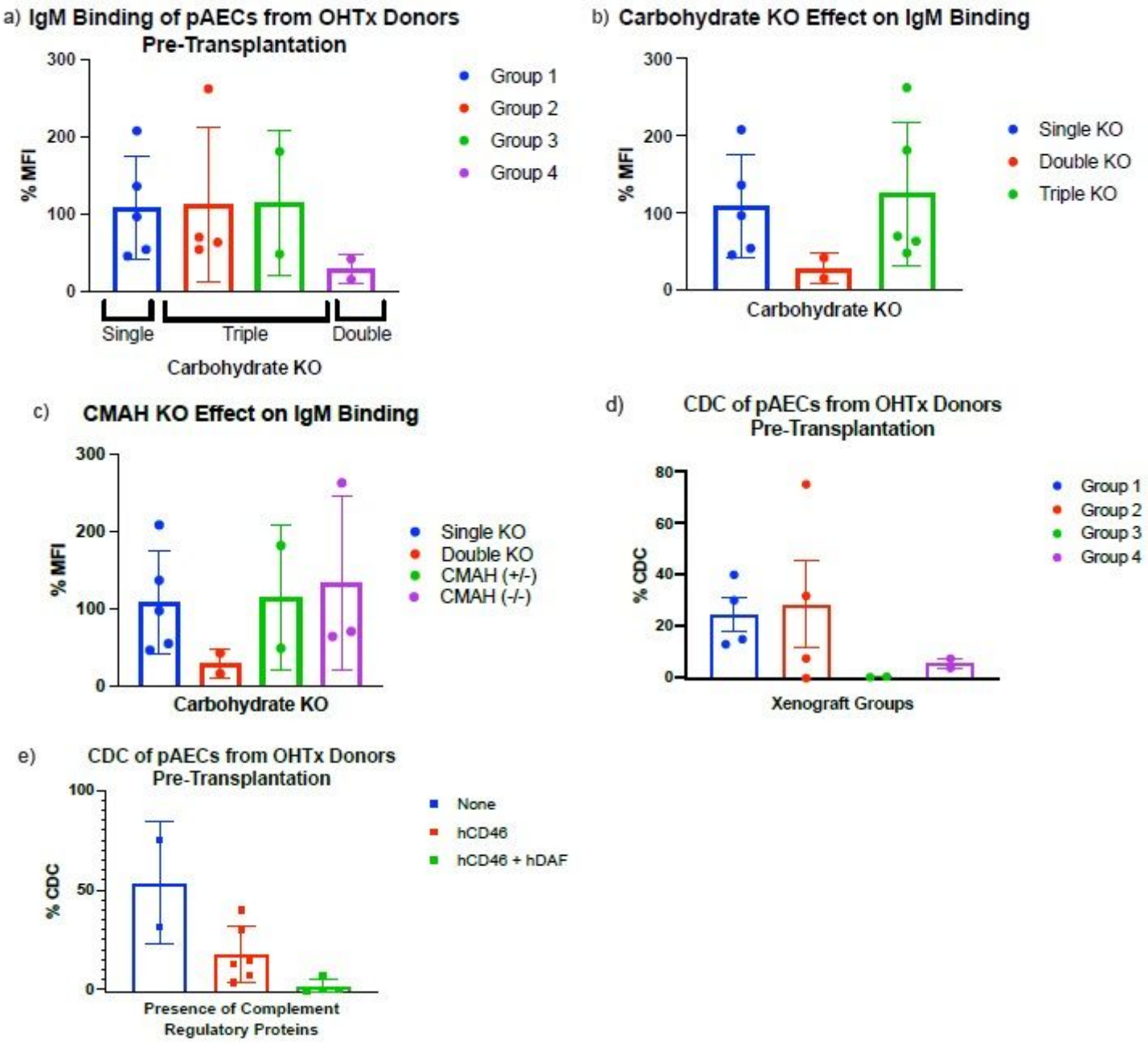

Figure 3

Characterization of multi-gene cardiac xenografts. a) IgM binding of pAECs from either xenograft donors or donor litter mates exposed to serum from Groups 1-4 recipients prior to OHTx. b) IgM binding from panel a, grouped by single, double or triple KO xenografts. c) IgM binding from a and b, grouped by CMAH (+/-) vs. (-/-). Single= GGTA1KO, double= GGTA1KO and B4GalNT2, triple=GGTA1KO, B4GalNT2 and CMAHKO. %MFI=MFI as a percent of control. CDC and IgM binding were performed as triplicates and presented here as an average of triplicates. d) complement dependent cytotoxicity (CD C) measured on pig aortic endothelial cells (pAECs) from either xenograft donors or donor litter mates exposed to serum from Group 1-4 recipients prior to orthotopic transplantation (OHTx). e) CDC from panel d, grouped by complement regulatory proteins hCD46 and hDAF.

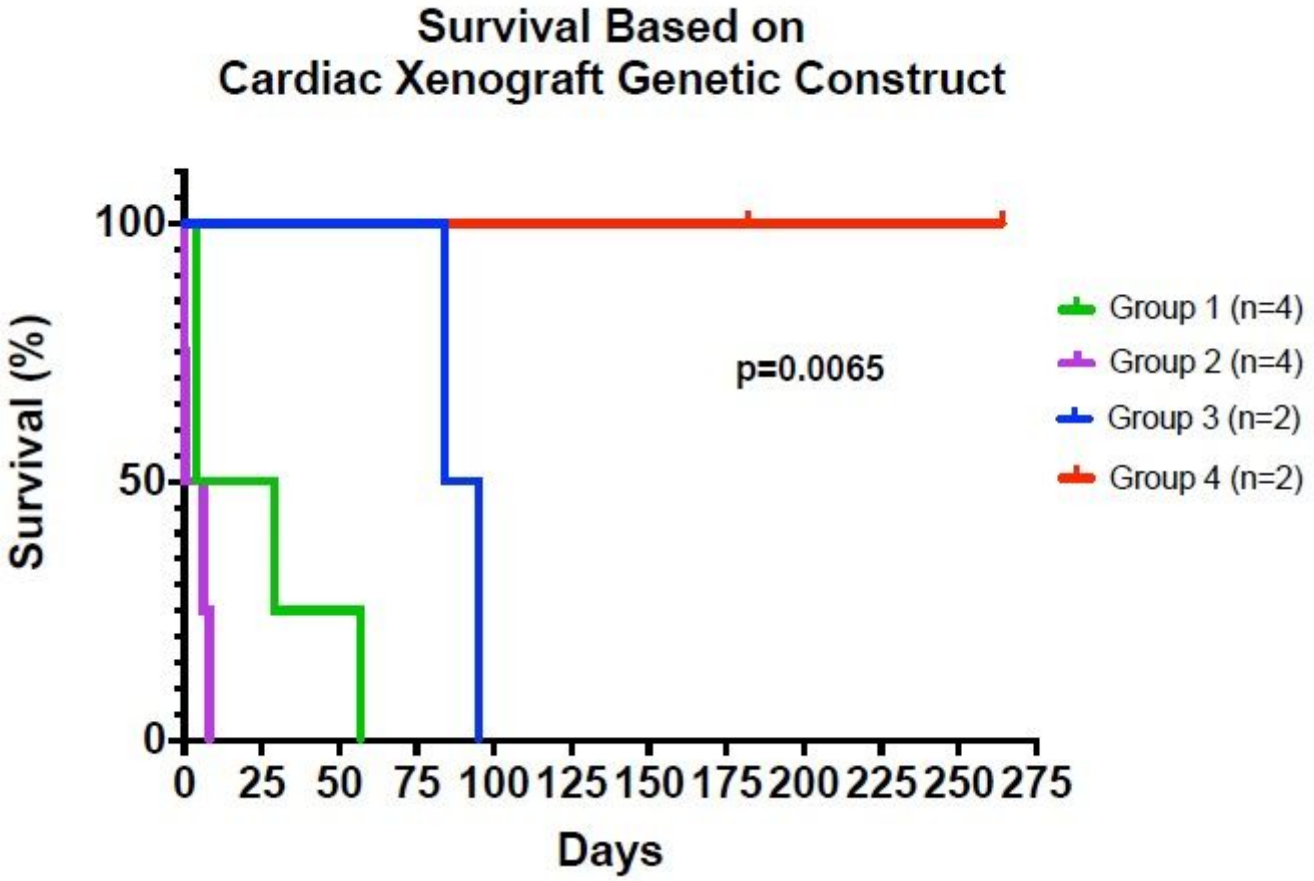

Figure 4

Recipient survival of Groups 1-4. Survival defined as time after transplantation before requiring euthanasia for deteriorating condition. Group 4 deaths censored for lack of evidence for immunologic rejection seen on histology, contributing to recipient deterioration. All other grafts contained histologic evidence of cardiac abnormalities contributing to deterioration requiring euthanasia. P=0.0065 by Log-rank (Mantel-cox) test, suggesting a significant difference in survival between Groups 1-4.

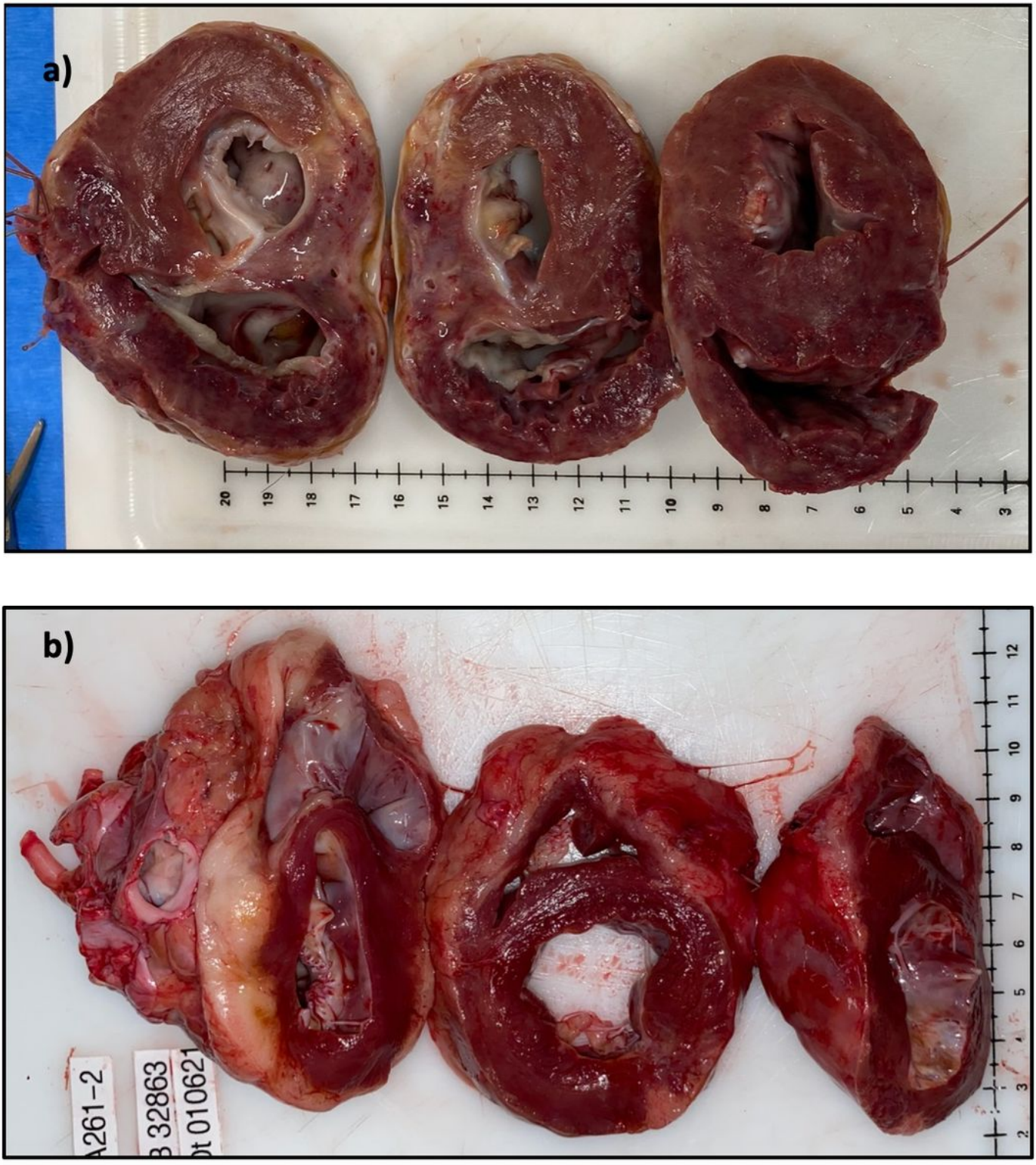

Figure 5

HRKO versus non-GHRKO xenografts. a) non-GHRKO grafts (Group 3) exhibited biventricular wall thickening. Here, B33121 survived 84 days prior to requiring euthanasia for symptoms of diastolic heart failure. b) GHRKO graft (Group 4) exhibiting normal histology without thickening at 182 days post-transplantation. T his animal (B32863) waseuthanized for weight loss as required by our institutional animal care committee, but was exhibiting excellent graft function.

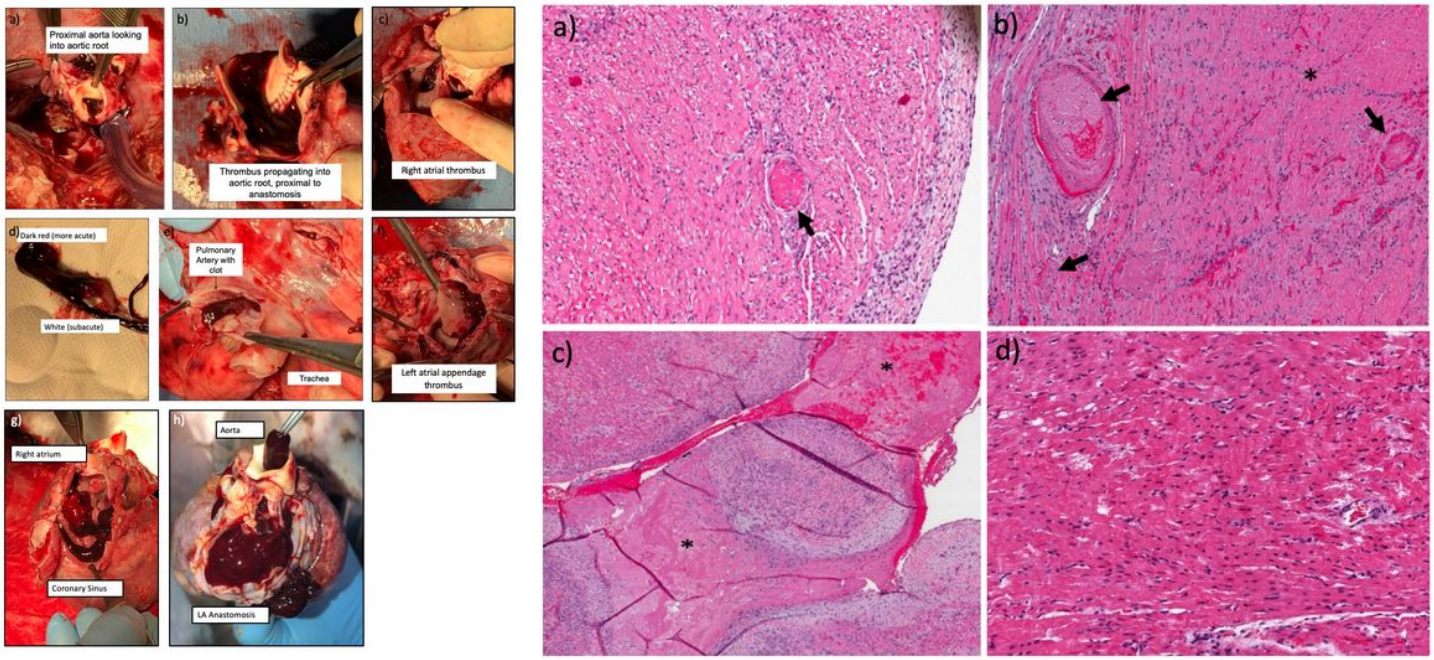

Figure 6

a: Thrombotic complications in Group 2 (xenografts without thromboregulatory proteins). panels a-f, showing B33167's xenograft at explantation. Consists of propagating thrombus of the aortic root (a, b and d), left and right atrial thrombus (c and f) and pulmonary artery (e). Pulmonary artery and left atrium appear to have acute and subacute components. intracardiac thrombosis of B33156 within coronary sinus (g), aorta and pulmonary vasculature (h). b: Histologic findings on H&E in Group 2 (xenografts without thromboregulatory proteins). a) B33167 right ventricle, 10x magnification. Fibrin thrombus (arrow) in a background of ischemic myocytes. b) B33156 apex, 10x magnification. Fibrin thrombi (arrows) and a region of ischemic myocytes (asterisk). c) B33156 left ventricle, 10x magnification. Note the intracardiac organizing thrombus (asterisk). d) B33060 right ventricle, 10x, note contraction bands and hypereosinophilia, indicating an early necrotic process.

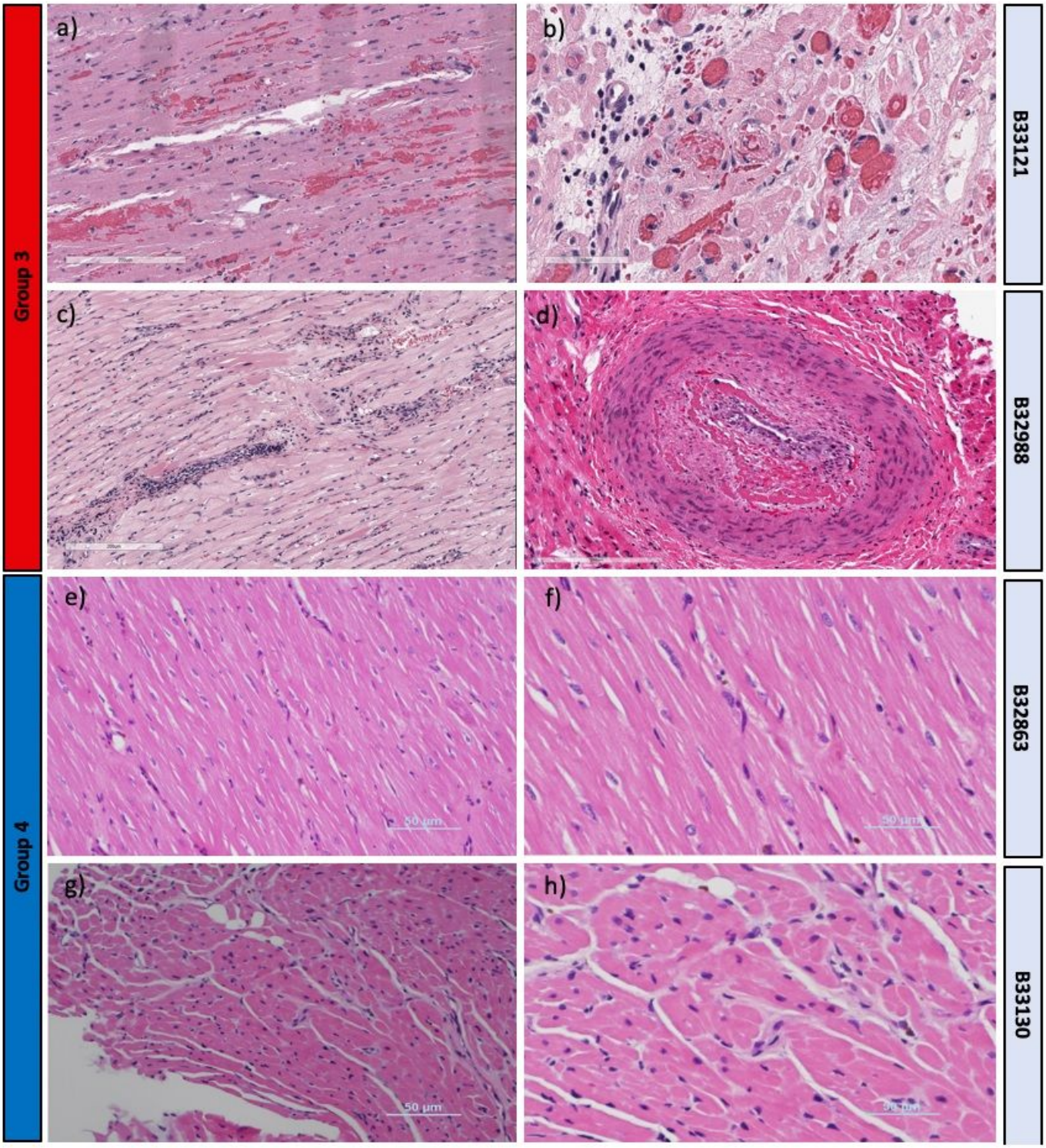

Figure 7

H&E in Long-term Survivors Between Groups 3 and 4. a) Group 3, B33121 LV (17X)- congestion, mild interstitial hemorrhage individual myofiber degeneration and necrosis, b) Group 3, B33121 RV (40X) veins with intravascular thrombosis c) Group 3, B32988 LV (14X) interstitial mononuclear lymphoplasmacytic inflammation, scant perivascular hemorrhage myodegeneration d) Group 3, B32988 RV (20X) organized thrombus in muscular artery, consistent with chronic xenograft vasculopathy e) Group 4, B32863 RV

(20X) normal myocardium without evidence of rejection f) Group 4, B32863 RV (20X) normal myocardium without evidence of rejection g) Group 4, B33130 RV (20X), endomyocardial biopsy, normal myocardium without evidence of rejection h) Group 4, B33130 RV (40X), endomyocardial biopsy, normal myocardium without evidence of rejection

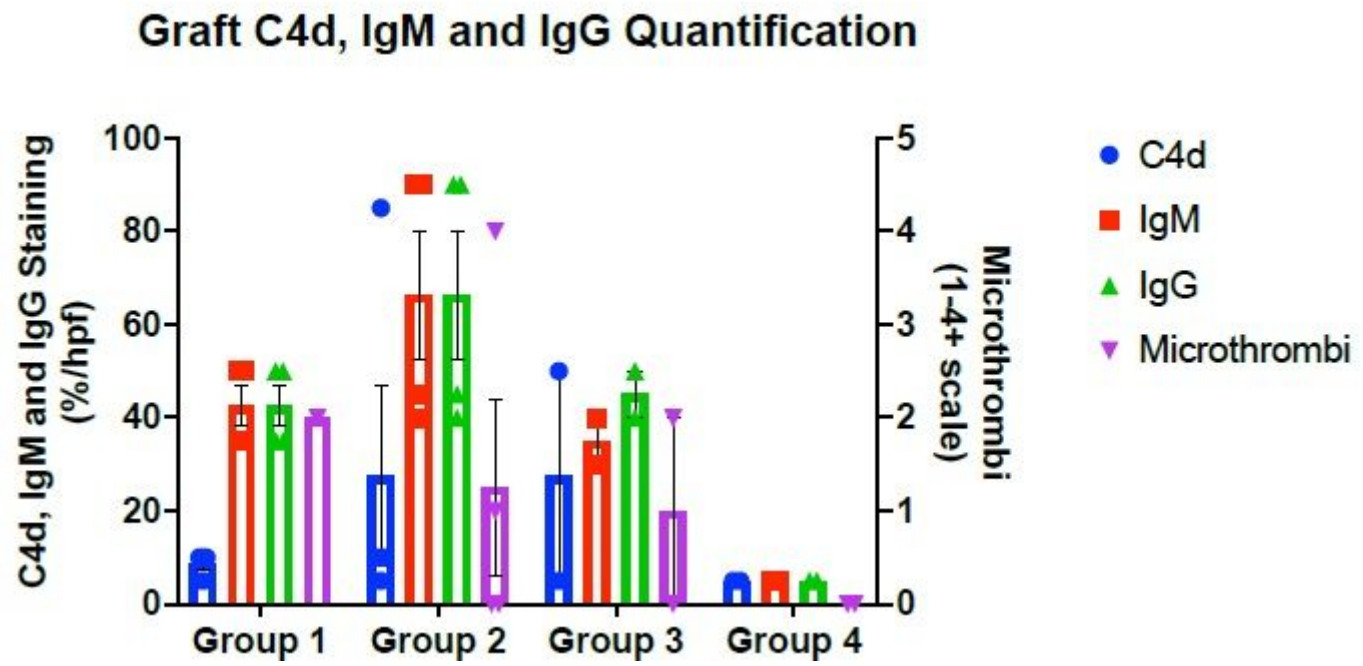

Figure 8

IHC quantification and microthrombi. Presented as averages within each group High power field was with 20x magnification, resulting in approximately an 870 micron field. Microthrombi were graded on a scale of 0-4+, where 1+ = >0 capillaries stained; 2+ = >1-5 capillaries stained; 3+ = >5-10 capillaries stained, 4+ = >10 capillaries stained, per high power field.

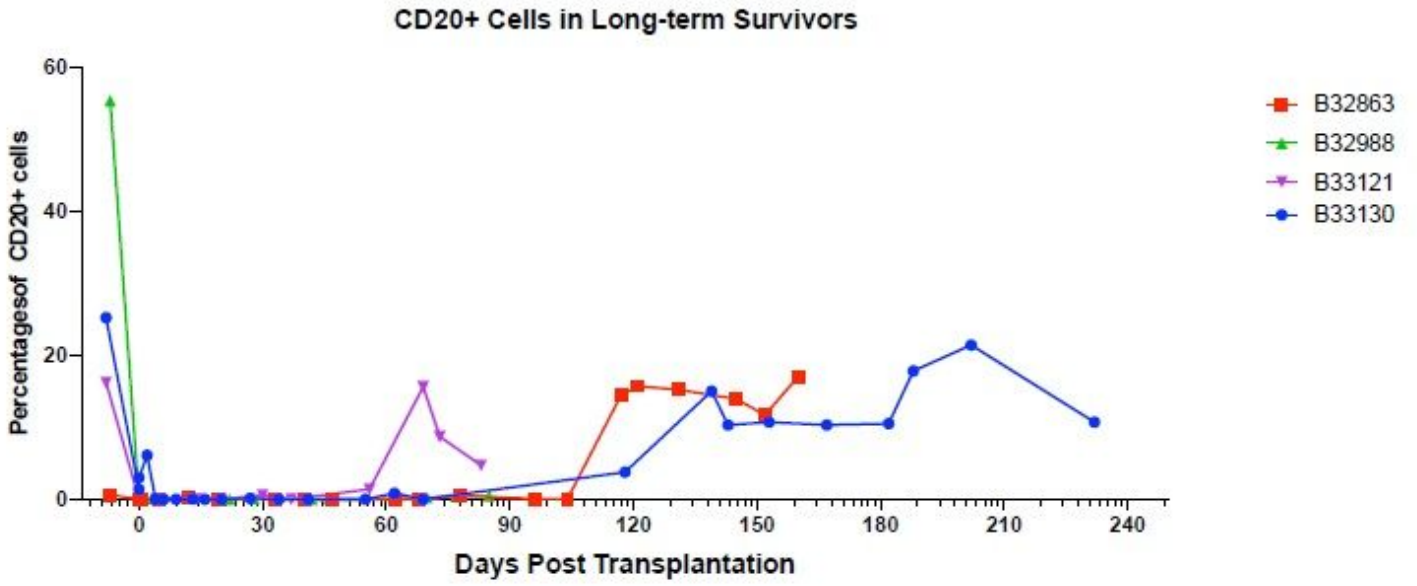

Figure 9

CD20+ cells in long-term survivors. B-cell depletion is adequate after induction therapy, followed by reconstitution by 60-90 days after transplantation. Percent CD20+ cells calculated as a proportion of total CD3+ cells. Group 3=B32988 and B33121; Group 4=B32863 and B33130.

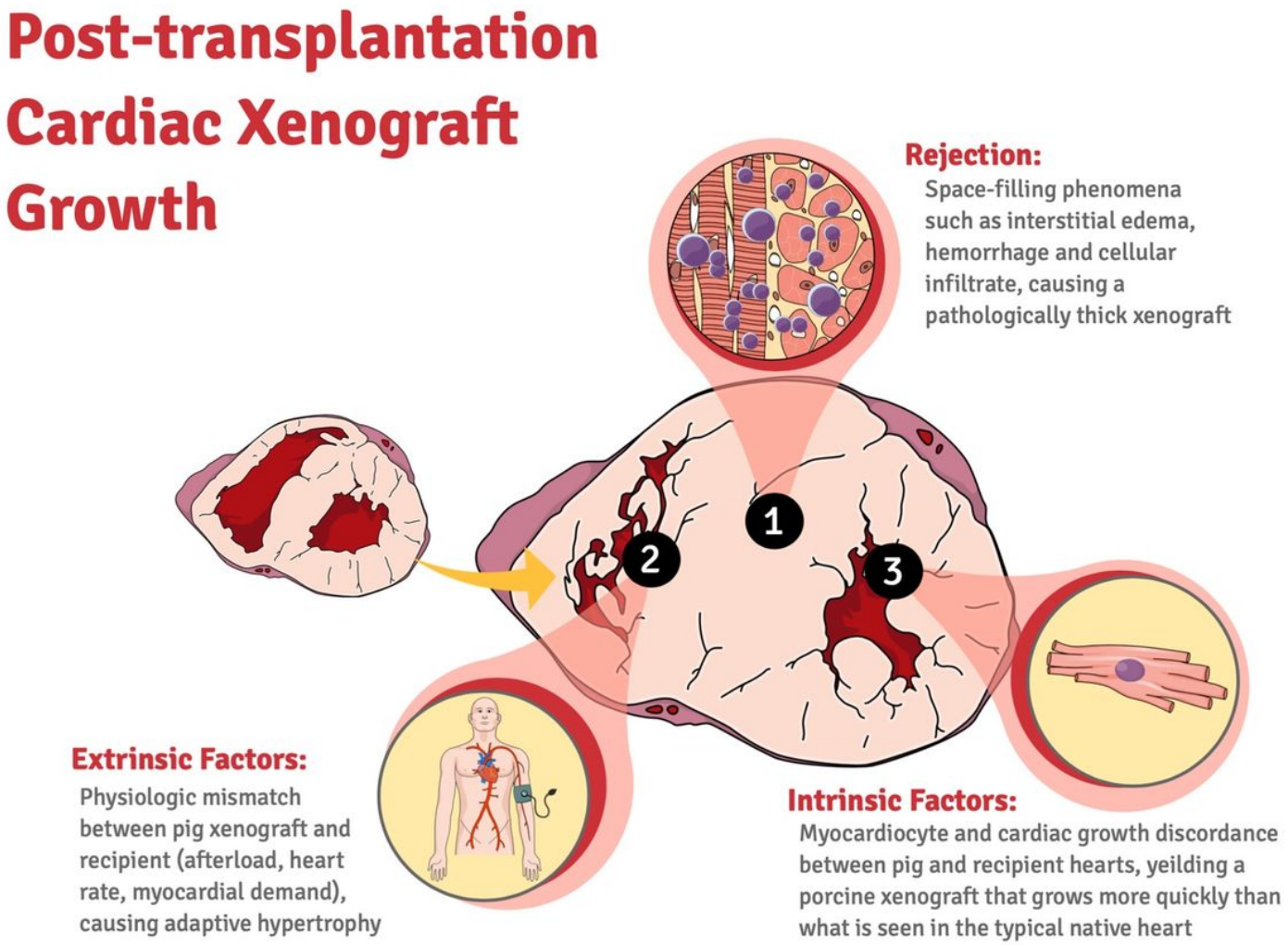

Figure 10

Potential mechanisms of post-transplantation cardiac growth in xenotransplantation. Post-transplantation cardiac xenograft growth is likely caused by both intrinsic and extrinsic factors, which includes rejection, intrinsic factors such as native xenograft growth. Other potential causes of growth include extrinsic factors such as physiologic mismatch leading to adaptive hypertrophy but was not observed in this study.

Supplementary Files

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- Video1.mov
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