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Bortezomib, C1-inhibitor and Plasma Exchange Do Not Prolong the Survival of Multi-transgenic GalT-KO Pig Kidney Xenografts in Baboons

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Abstract

Galactosyl-transferase knock-out (GalT-KO) pigs represent a potential solution to xenograft rejection, particularly in the context of additional genetic modifications. We have performed life supporting kidney xenotransplantation into baboons utilizing GalT-KO pigs transgenic for human CD55/CD59/CD39/HT. Baboons received tacrolimus, mycophenolate mofetil, corticosteroids and recombinant human C1 Inhibitor combined with cyclophosphamide or bortezomib with or without

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2–3 plasma exchanges. One baboon received a control GalT-KO xenograft with the latter immunosuppression. All immunosuppressed baboons rejected the xenografts between days 9 to 15 with signs of acute humoral rejection, in contrast to untreated controls (n=2) which lost their grafts on day 3 and 4. Immunofluorescence analyses showed deposition of IgM, C3, C5b-9 in rejected grafts, without C4d staining, indicating classical complement pathway blockade but alternate pathway activation. Moreover, rejected organs exhibited predominantly monocyte/macrophage infiltration with minimal lymphocyte representation. None of the recipients showed any signs of PERV transmission but some showed evidence of PCMV replication within the xenografts. Our work indicates that the addition of bortezomib and plasma exchange to the immunosuppressive regimen did not significantly prolong the survival of multi-transgenic GalT-KO renal xenografts. Non-Gal antibodies, the alternative complement pathway, innate mechanisms with monocyte activation and PCMV replication may have contributed to rejection.

Introduction

Xenotransplantation of wild type (WT) porcine vascularized organs in unmodified non-human-primates (NHP) leads to hyperacute rejection (HAR), mainly due to preformed natural xeno-antibodies (XNA). Since these XNA activate the complement cascade, genetically modified pigs expressing human complement regulatory proteins (hCRP) have been generated (1–3) and the organs of these mutant swine were efficiently protected against HAR (1). After the identification of the major xenoantigen Galactose-α-1,3-Galactose epitope (Gal) (4), other pigs lacking Gal expression were generated by knocking out the corresponding galactosyl-transferase gene (5–9). The use of these GalT-KO organs in pig-to-NHP heart (10, 11) and kidney (11–16) xenotransplantation resulted in prevention of HAR and modest prolongation of graft survival using standard immunosuppression. The survival of xenogeneic kidneys was inferior to that of heterotopic hearts, with graft losses characterized predominantly by thrombotic microangiopathy and acute humoral rejection (AHXR) involving Ig and complement deposition (10, 11, 16). However, in one series using a protocol directed toward T cell tolerance, Yamada et al. (15) achieved survival of composite thymo-kidney transplants up to 3 months, without substantive features of rejection and with no porcine cytomegalovirus (PCMV) infection evident (11, 15).

More recently, new strains of pigs combining multiple genetic modifications have been generated. Hearts from GalT-KO pigs transgenic for hCD55 (17) or hCD46 (18, 19) grafted heterotopically into baboons survived from 15 to 52 days (17), and up to one year with recipient B cell-depletion (18, 19). In the present study, we performed, for the first time, xenotransplantation of kidneys from GalT-KO pigs multi-transgenic for human CD55, CD59 to target complement, CD39 to modulate purinergic signalling and thrombosis and H-transferase (α1,2-fucosyl-transferase, HT, initially to diminish Gal expression), in a life-supporting model in baboons in order to evaluate whether these additional genetic modifications would confer further advantage to GalT-KO pig organs. As adjuncts to conventional immunosuppression, recipients also received combined experimental regimens including plasma exchanges, recombinant human C1 inhibitor (rhC1-INH) and bortezomib to block complement activation and XNA producing B/plasma cells respectively. The

potential for porcine endogenous retrovirus (PERV) transmission and the possible PCMV/BCMV activation were also tested.

Materials and Methods

Animals, xenotransplantation procedure and immunosuppressive treatments

Genetically modified pig (*Sus scrofa*) kidneys were grafted into baboon recipients (*Papio anubis*, originating from the Centre National de la Recherche Scientifique (CNRS)-primatology centre, Rousset, France) as described (13). All pigs studied were generated by Somatic Cell Nuclear Transfer (SCNT) (20).

Four experimental groups were studied according to the treatment and/or the donor source. All recipients were splenectomized at the time of transplant, except for 2 baboons from group#1 (Table 1). Clinical parameters and renal function were monitored daily by measurement of plasma creatinine, BUN, blood biochemistry and diuresis.

The NHP groups are described in detail in Table1. In brief, NHP from the control group and groups#1–3 received kidney transplants from GalT-KO.hCD55.hCD59.hCD39.hHT*Tg* pigs, originally generated by Cowan *et al*. (2, 9, 21, 22), (the triple-transgenic pigs were made before it was possible to knock out Gal, and HT was included in an attempt to reduce Gal; the HT transgene cannot be separated from CD55 and CD59 because they are co-inherited), whereas kidneys grafted in baboons from group#4 came from GalT-KO pigs initially generated with the genetic background of MGH pigs (8) (Table 1). To study anti-pig immunization, 2 untreated recipients were kept alive after rejection: one autologous kidney was kept during surgery with a clamped ureter (under a powerful analgesia) to be unclamped at rejection and graft removal. All other recipients underwent a bilateral nephrectomy prior to transplantation.

Group #1 recipients (n=4), received a combined treatment modified from our previous studies using hCD55 transgenic donors (13, 23), consisting of rhC1-INH (kindly provided by Pharming (the Netherlands) already used in NHP (24)) at 200U/Kg/8hrs for 5 days, cyclophosphamide at 40 mg/Kg at days (d) −1 and d0, 20 mg/Kg at d2 and 10 mg/kg at d4, mycophenolate mofetil at 70 mg/kg/day, tacrolimus at 0.2 mg/Kg/day, and corticosteroids at 1 mg/Kg/day for the duration of the xenotransplantation. In group #2 (n=4), two plasma exchanges (PE) were added at d-4 and d-1 to remove preformed circulating non-Gal XNA, then plasma was replaced by human serum albumin. In addition, bortezomib, 1.3 mg/m^2 (Janssen Cilag Int.), was given instead of cyclophosphamide at d-17, −14, −10, −7, −4, −1 and d7 (dose and timing were determined according preliminary pharmacodynamics experiments shown in supplementary data). These recipients received also rhC1-INH at 200U/Kg/8hrs (n=2) or 400U/Kg/8hrs (n=2) from d5 to d9. Group #3 (n=4) and #4 (n=2) recipients received the same treatment as those of group #2 with minor modifications: rhC1-INH was given at 400U/Kg/8hrs from d4 to d8 and an additional PE was performed at d3 (Table1). All treatment modifications between the groups were introduced on the basis of results obtained from the previous group (see results and discussion).

The French regional ethical committee for animal experimentation approved the study. The pig SCNT work was conducted in conformity with the Italian legislation DLg n116/92 and approved by the local ethics committee for animal experimentation of Avantea.

Histological analyses

All tissue sections were stained with H&E and examined in a blinded manner. Immunofluorescence (IF) was performed on biopsy and graftectomy sections of 10 μm fixed in acetone. Sections were incubated overnight at 4°C with *Bandeiraea simplicifolia*- Isolectin B4 (IB4)-FITC (Sigma, Saint-Louis, MI, USA), mouse anti-hCD55 (67), mouse anti-hCD59 (MEM-43), mouse anti-hCD39 (A1), rabbit polyclonal anti-hIgM all from AbDSerotec (Oxford, UK), rabbit anti-hCD3, mouse anti-hCD20 (L26), mouse anti-hCD68 (KP1), rabbit anti-hC3c all from Dako (Glostrup, Denmark), mouse anti-hCD4 (13B8.2) mouse anti-hCD8 (B9.11), mouse anti-hCD11b (BEAR1) all from Beckman Coulter (Fullerton, CA, USA), mouse anti-hC4d (Quidel, San Diego, CA, USA) and mouse anti-hC5b-9 (aE11, Diatec, Oslo, Norway). After washing, sections were incubated with either AlexaFluor®568-labelled goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) or FITC-labelled donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) for 90 min. at room temperature (RT). Sections were analyzed using an Axioskop2 plus microscope and Axion Vision software (Zeiss, Le Pecq, France).

Thrombin generation measurements

Thrombin generation (TG) was measured by the Calibrated Automated Thrombogram method in Platelet Poor Plasma (PPP) (25). Briefly, PPP was mixed at 4:1 in PPP-Reagent (Thrombinoscope BV, Maastricht, The Netherlands), with Tissue Factor and synthetic phospholipids (respectively 5pM and 4μM final concentration). The reaction was initiated by adding a fluorogenic substrate (Z-Gly-Gly-Arg-AMC, Thrombinoscope BV) and CaCl2, and fluorescence was read in a Fluoroscan Ascent reader (Thermo Labsystems, Helsinki, Finland). TG curves were calculated using the Thrombinoscope Software and Thrombin Calibrator (Thrombinoscope BV). Prolongation of Lag-times and lower values of both peak thrombin and ETP are indexes of hypocoagulability.

Donor Screening and XenoAntibody Monitoring by FlowCytometry

Confirmation of gene inactivation (KO) and transgene expression were performed on resting human (HAEC), WT and donor porcine aortic endothelial cells (PAEC) isolated from aorta (26, 27). Briefly, 1 to 2×10^5 PAEC after 2 washes in cold FACS buffer and incubated for 30 min. at 4°C with IB4-FITC, mouse anti-hCD55 (67), mouse anti-hCD59 (MEM-43) and mouse anti-hCD39 (A1) (AbDSerotec). Thereafter, cells were washed twice and incubated with anti-mouse IgG-FITC (for 30 min. at 4°C).

The baboon serum xenoreactivity was assessed by flow endothelial cross-matches in donor PAEC. Recipient sera were harvested at d-17, d-4 pre- and post-PE, d-1 pre- and post-PE, d0, d1, d2, d3 pre- and post-PE, d4 and weekly thereafter. Heat inactivated sera were incubated for 30 min at RT with 1 to 2×10^5 PAEC, thereafter cells were washed twice in cold FACS buffer and incubated separately for 30 min at 4°C with rabbit polyclonal anti-

hIgM or anti-monkey IgG-FITC (AbDSerotec). The anti-pig IgM was revealed using donkey anti-rabbit IgG-FITC (Jackson ImmunoResearch).

Baboon serum cytotoxicity was measured by Flow Cytometry Complement-Mediated Cytotoxicity Assay. Briefly, heat inactivated sera were incubated for 30 min. at 4°C with 1 to 2×10^5 donor PAEC, thereafter the cells were washed twice in FACS buffer and incubated for 30 min at 37°C with 1:3 diluted rabbit complement. Then, cells were washed and propidium iodide was added to detect dead cells.

Cells were washed, resuspended in FACS buffer and analyzed using a Canto-II Flow cytometer with DIVA (Becton Dickinson, San Diego, CA, USA) and FlowJo software (Tree stars, Ashland, OR, USA).

Serum Complement monitoring

Classical hemolytic complement (CH50), alternative pathway complement (AP50), mannose-binding lectin complement (MBL50) activities, some complement components (C3, C4 and MBL) and C1-Inhibitor were measured in pre- and post-xenotransplantation plasma samples from control and groups#1–3 recipients. Due to the cross-reactivity of human reagents with baboons, we used human activation and detection systems (28–31).

PERV, baboon and porcine CMV detection

Serum samples were taken from control animals pre-xenograft, day 41, and month 3 (euthanasia); and treated animals prior xenograft and at euthanasia. Viral RNA (vRNA) was extracted from serum using the QIAmp viral RNA mini kit (Qiagen, Crawley, UK). The presence of PERV RNA was detected by RT-PCR using a method modified from that described by Paradis *et al* (32). To determine if organ recipients presented microchimerism, PBMC isolated from the recipient and donor's blood and tested for the presence of porcine centromeric DNA and PERV as described previously (32). A PERV:centromeric ratio higher in the recipient than donor would indicate PERV integration and thus possible infection.

DNA was isolated from donor PBMC, donor spleen, recipient baseline PBMC, recipient date of death PBMC and the xenograft, using the DNeasy mini kit (Qiagen). DNA isolated from recipient and xenograft tissue was screened by qPCR for the presence of BCMV. DNA isolated from donor animal, recipients and xenografts were screened by qPCR for the presence of PCMV. Both the PCMV and BCMV qPCR assays were modified from the methods described by Mueller *et al* (33). The sensitivity of the assay is 10 (BCMV) and 1 (PCMV) copies per reaction (Figure S1).

Results

Knockout and transgene evidence in pig kidneys

The lack of Gal and the expression of hCD55, hCD59 and hCD39 transgenes were assessed by pig kidney IF staining (Fig. 1A) and by FACS on resting PAEC and HAEC (Fig. 1B). Both analyses confirmed the absence of Gal, strong expression of hCD55 and hCD59, and weak levels of expression of hCD39 in PAEC and pig kidneys.

Survival, histological analyses and coagulation data

In the untreated control group, no HAR occurred but graft survival did not exceed d4, because of biopsy proven acute vascular rejection (Fig. 2a) indicating persistent roles for XNA despite expression of hCRP transgenes. Three grafts (one each within groups #1, 2 and 4) were lost due to technical failure within 4 days, whereas the remaining eleven recipients rejected their xenografts between d9 and d15 (median 12 days; mean 12 days) (Table 1).

The graft survival of the remaining GalT-KO kidney (group #4) was 11 days and did not differ from GalT-KO.hCD55.hCD59.hCD39.hHT*Tg* kidneys survival in groups #1–3.

In all rejected grafts from groups #1–4, histology showed signs of acute vascular rejection with focal interstitial haemorrhage, glomerular and capillary thrombi, interstitial cellular infiltrate and oedema qualified as AHXR and ACXR (Fig. 2).

Usually animals showed an immediate graft function (assessed by the creatininemia), except the ones with surgical complications (V893AA, O22VB, PA936F, PA936G & K24E) that could be fixed (Fig. 3).

Global coagulation data showed at ultimate time point of rejection in groups #2–3 (Fig. 4A), a reduction of thrombin generation presumably due to coagulation factors consumption secondary to the immune microvascular aggression, as already described (34). Related thrombocytopenia were also noted at the same time (Fig. 4B), suggesting a possible ultimate rising consumptive coagulopathy although no diffuse haemorrhages were noted at the autopsy.

Immuno-staining analyses

Immuno-staining showed IgM deposition within the graft from d3 post-transplantation in both vascular and glomerular areas in control animals (Fig. 5Aa) as well as in immune-suppressed animals, without (Fig. 5Ab) or with (Fig. 5Ac, d, e & f) multiple PE. This suggests that even a small amount of preformed circulating anti-non-Gal Ab rapidly bound microvascular structures of the xenograft and induced severe and early damage. C3c, C4d and C5b-9 deposits were observed, with a similar Ig deposition kinetics in animals before (Fig. 5Aa) and after the end of rhC1-INH treatment (Fig. 5Ab: day 15; e: day12; f: day11). Interestingly, we observed a C3c and C5b-9 deposition, without C4d staining, at d4 in a functioning graft (Fig. 5Ad) while the recipient #PA936G was still under rhC1-INH, suggesting an efficient complement classical pathway blockade as long as the rhC1-INH was given but a persistent alternative pathway.

At the cellular level, we observed a modest B cell infiltration (CD20) in perivascular areas in control and group #1 animals (Fig. 5Ba & b), whereas this infiltrate was almost abrogated in biopsies of animals from groups #2 (Fig. 5Bc), #3 (Fig. 5Bd & e), and #4 (Fig. 5Bf) suggesting an efficient B cell blockade by bortezomib. A marked CD4+ T cell infiltrate in perivascular areas was observed in controls, as opposed to treated animals for which it remained weak. CD8+ T cell infiltrate was minor in all groups. Interestingly, we observed in all groups an early intense and dominant monocyte/macrophage infiltration (CD11b, CD68) in perivascular areas as well as inside glomeruli (Fig. 5Ba, b, c & e), proving an important

innate cellular response but also suggesting a possible direct or indirect binding to the porcine endothelium in GalT-KO xenotransplantation rejection.

Serum xenoreactivity, cytotoxicity and complement monitoring

The two control animals, kept alive after rejection, were followed to monitor their long term immunization. The low level of preformed anti-non-Gal IgM XNA decreased further during the first days post-transplant, most likely due to binding to the graft. Thereafter, anti-non-Gal IgM and IgG increased respectively from d6 and d9 to reach a maximum around d21 and d28 and then decreased slowly (Fig. 6A). These kinetics were similar to group #1 recipients, with a maximum of immunization at the time of rejection. The use of bortezomib in the pre-transplant period (groups #2–4) induced a modest reduction of preformed IgM and IgG (data not shown). Because of the evidence of preformed anti-non-Gal Ab, PE were then added to bortezomib (groups #2–4) and efficiently decreased these antibodies, followed by a fast rebound, justifying repetition until an optimized Ab depletion; thereafter, half of the baboons showed no re-increase of XNA (d-1 group #2, d3 groups #3–4). Serum complement dependent cytotoxicity (CDC) persisted and increased at the time of rejection in most cases (Fig. 6B), suggesting a possible role of high affinity anti-non-Gal Ab in the early graft damage leading to AHXR. However, other than early alternative pathway activation, plasma complement monitoring showed no major change of the classical and MBL pathways (data not shown), suggesting that the complement activation took place in the xenografts.

PERV, baboon and porcine CMV detection

PERV vRNA levels found in the recipient's serum along with the recipients and donors PERV:centromeric ratios are shown in Table 2. With the exception of animals #PA997C and #PA936G, all recipients produced PERV:centromeric ratios lower than that of their donor animal. This would indicate that PERV vRNA detected in their serum and PERV DNA detected in their PBMC preparations was a result of microchimerism (circulating donor cells) and not PERV infection. The PERV:centromeric ratios observed in animals #PA997C and #PA936G are slightly higher than their donors' PERV:centromeric ratios. However, these values were not significantly different (p=0.315 and p=0.227 respectively).

All recipient baboons testing positive for BCMV at baseline (n=3) showed an increase in BCMV copy number at time of rejection; (23–253 copies/500ng DNA (mean 107.3 copies/500ng DNA) at baseline vs. 79–3194 copies/500ng DNA (mean1181 copies/500ng DNA) at rejection). In addition 2 baboons (#V907J and #PA997C), which tested negative for BCMV at baseline tested positive for BCMV at rejection. No BCMV infection was detected in any of the xenografts (Table 3).

Of the 7 donor animals used, PCMV was detected in the blood (n=3) and in the spleen (n=4). PCMV detection in these animals was also noted in the donor kidney xenograft (Table 3). The two donor animals for group #3 were positive at time of graft and PCMV was also detected in the blood of the recipient animals at the time of rejection. Of the recipients of positive donors used for group#2, only 1 animal was shown to have a low copy number of PCMV at the time of rejection and one was negative (Table 3). In most of PCMV positive kidney xenograft, we have observed early tubular cell nuclear dystrophia potentially related

to this viral replication, but in any case we observed specific signs of CMV kidney aggression, such as Cowdry inclusions (data not shown).

Finally, the Table 4 summarized most results obtained in this study, indicating for each group the graft survival (days), the cause of graft failure, Immuno-histology at rejection, the nature and cytotoxicity of peripheral elicited anti-nonGal Ab, and the viral PERV, BCMV and PCVM status.

Discussion

This study reports for the first time pig-to-primate renal xenotransplantation using GalT-KO.hCD55.hCD59.hCD39.hHT*Tg* donors. In no case did we observe HAR, confirming the efficiency of the Gal KO strategy (10, 15, 16, 18, 19). Unfortunately, CD39 expression was not noted at high levels and furthermore because of only one control baboon grafted with a GalT-KO donor, we could not assess whether the efficient combined GalT-KO/CRP transgene strategy was of benefit.

In fact, cases of HAR have been reported in GalT-KO cardiac xenotransplantation, but never using GalT-KO-hCD55*Tg* donor (17), suggesting that anti-non-Gal Ab dependent activation of complement may be beneficially regulated by local CD55 expression only at early times. However, the major issue is that survival times observed in our study are not as encouraging as earlier experience in this area (specifically with GalT-KO thymokidneys in PCMV free studies), irrespective of the modified immunosuppressive strategies. Indeed, complement and Ab producing cell blockade, PE and general immunosuppression all failed to prevent AXHR and AXCR, as noted previously for GalT-KO heart (11, 17, 35) and kidney (15, 16) xenotransplantation.

Our results confirm the presence of circulating preformed cytotoxic anti-pig-non-Gal IgM and IgG in baboons, already demonstrated by others (11, 15–17). The rapid occurrence and the cytotoxic property of these elicited anti-non-Gal Ab also suggest their significant involvement in AHXR. Therefore, we also employed the anti-B/plasma cell agent bortezomib to decrease active bone marrow plasma cells, but only a modest reduction of circulating preformed IgM/G XNA was achieved similar to an earlier report (36). Pre- and post-xenotransplantation PE efficiently reduced circulating preformed XNA, although they were followed consistently by a rebound. Our results suggest that this treatment was efficient at blocking the B/plasma cell reactivity in some animals but that even when it did, it could not control the intra-graft aggression. In this regard, other drug combinations have been reported to result in graft survival of heterotopic heart xenotransplants for up to 1 year without XNA recovery (18, 19) and a combination of drugs and a thymokidney have led to survivals up to 83 days (in PCMV free animals) (37).

Structural identification of non-Gal Ag in the pig-to-baboon combination is also an important objective. In human, recent data argue for a role of anti *N*-glycolylneuraminic acid (Neu5Gc) antibodies (38, 39) and double GalT and Neu5Gc-KO pig (40) have been generated which are less sensitive to human serum CDC. However, our data (supplementary data) have demonstrated considerable variability in the anti-pig carbohydrate antibody

repertoire between primates, limiting the general conclusions about the importance of particular carbohydrate antigens in NHP xenotransplantation trials.

One potential advantage of our pig donors was the combination of hCRP (CD55, CD59). The protection against complement activation seemed to play a role only in the early post-transplant period and justified the use of rhC1-INH thereafter, as shown in alloAb-mediated rejection (24). rhC1-INH blocked efficiently of the classical pathway activation only as long as it was used but failed to prevent eventual xenotransplantation rejection, possibly due to alternative pathway activation. This phenomenon described in xenotransplantation rodent models (41), was not previously demonstrated in NHP and might justify a strategy of wider complement blockade such as anti-C5 mAb.

As shown in GalT-KO heart (11, 35) and kidney (11) xenotransplantation, we observed a strong monocyte/macrophage infiltrate in all groups, regardless of the immunosuppressive treatments. Interestingly, this infiltrate affected glomerular capillaries, participating and/or worsening vessel damage and possibly linked to deposition of complement factors such as iC3b, known to bind to CD11b/18 (CR3). This role of macrophages in the xeno-cellular response may support the use of the inhibitory pathway of CD47-SIRPα through a transgenic approach (42). Obviously in our model, T and B cell infiltrate remained minor, although the majority of animals developed elicited XNA requiring a T-B cooperation in peripheral lymphoid organs, suggesting a suboptimal immunosuppression.

As already known (34), late time points of rejection were associated to coagulation disorders potentially leading to consumptive coagulopathy, suggesting that genetic modified organs did not bring any protection in this regard without ruling out that they could be worse.

The recipients showed no evidence of PERV infection, despite the use of strong immunosuppression. Thus, the potential increased risk of PERV transmission in the Gal-KO context, hypothesized on the basis of the absence of a possible protective effect of Gal Ab toward Gal negative PERV (43), was not observed here.

Similarly, despite an increase in BCMV copy number in the serum of some immunosuppressed animals, none showed signs of BCMV disease and all xenografts were BCMV-negative. The copy number of PCMV, which can play a role in the rejection of porcine renal xenografts in NHP recipients (33, 37, 44, 45), was low in donors pre-transplant, comparable to other studies (46). However, a significant increase in intra-graft copy number was observed post-transplant. PCMV replication was much higher in group#3 than in group#2, potentially due to the additional PE in group#3, although no Cowdry inclusions indicative of active PCMV graft infection were observed. Detection of circulating PCMV in the blood of the recipients at time of rejection could be explained by the presence of porcine cell microchimerism.

One question to be clarified is whether the GalT-KO.hCD55.hCD59.hCD39.hHT*Tg* phenotype may become a disadvantage regarding protection from CMV, specifically CD55 and CD59 and incorporation into viral envelope limiting lysis (47). Another question is whether PCMV infection may have caused graft loss too soon for the benefit of the other genetic modifications to have exerted a measurable effect using the immunosuppressive

regimens we have tested. This possibility would be supported by recent studies of Yamada and colleagues showing marked improvement of renal xenograft survival by use of PCMV-free donors, using an immunosuppressive regimen including costimulatory blockade with anti-CD154 mAb (48, 49). Thus, further attention to PCMV reactivation, further genetic modifications and further modifications of immunosuppression may be needed to achieve long-term xenograft survivals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACXR	Acute Cellular Xenograft Rejection
AHXR	Acute Humoral Xenograft Rejection
AlloAb	Allo-antibodies
AP50	Alternative pathway complement activity
BCMV	Baboon Cytomegalovirus
CH50	Total complement activity
rhC1-INH	recombinant human C1 Inhibitor
CNRS	Centre National de la Recherche Scientifique
hCRP	human Complement Regulatory Proteins
Gal	Galactose-α-1,3-Galactose
GalT-KO	Galactosyl-transferase knock-out
HAR	Hyper Acute Rejection
hHT	human H-transferase (α1.2-Fucosyl-transferase)
IF	Immunofluorescence
MBL	Mannose-binding lectin
MBL50	Mannose-binding lectin pathway complement activity
NHP	Non Human Primates

PAEC	Porcine Aortic Endothelial Cells
PCMV	Porcine Cytomegalovirus
PE	Plasma Exchange
PERV	Porcine Endogenous Retrovirus
PPP	Platelet Poor Plasma
RT	Room Temperature
SCNT	Somatic Cell Nuclear Transfer
TG	Thrombin generation
WT	Wild Type
XenoAg	Xenoantigens
XNA	Xeno Natural Antibodies

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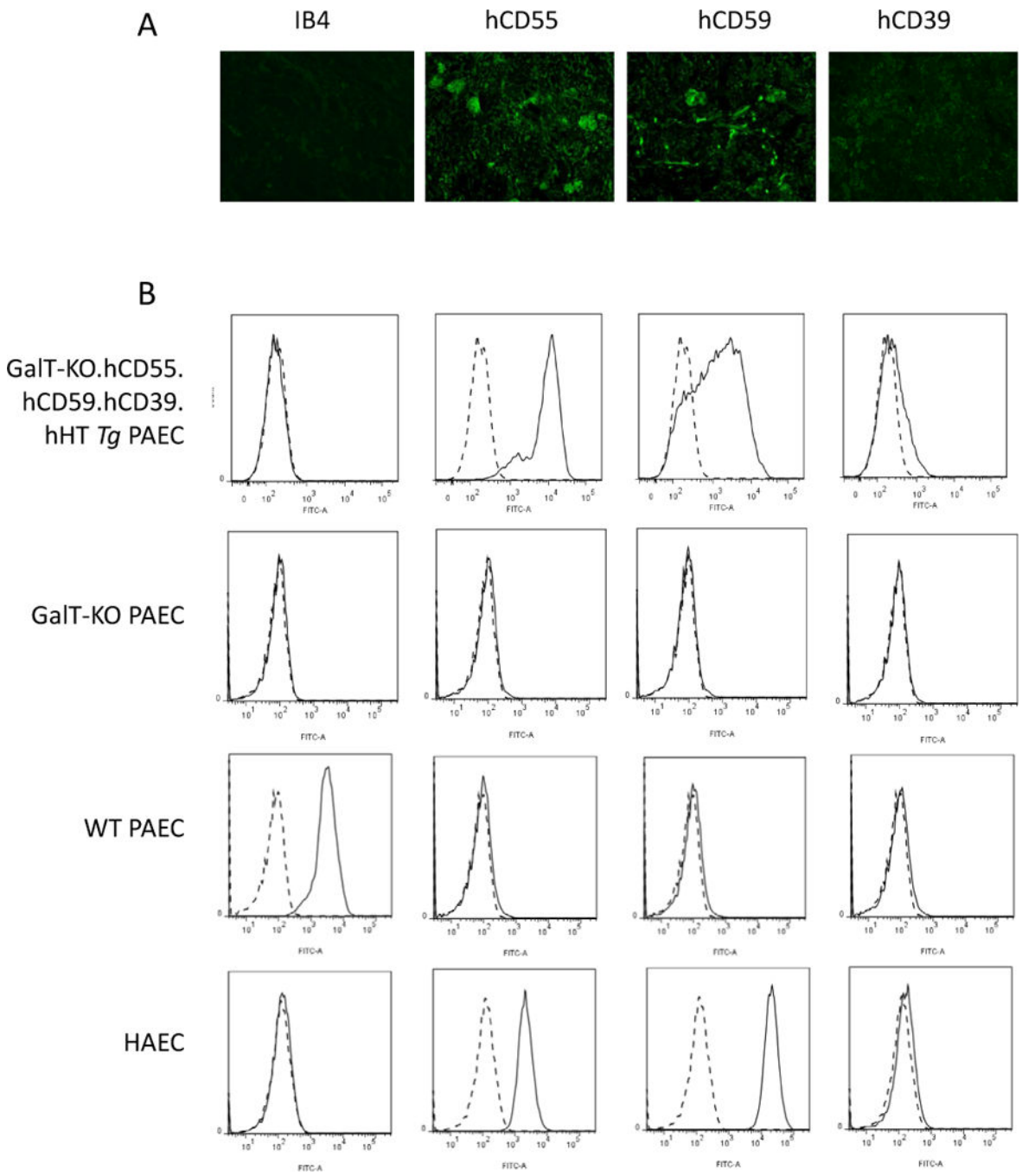


Figure 1.

Transgene expression and knock-out in pig cells and tissue. The expression of Gal epitope (IB4), hCD55, hCD59 and hCD39 was assessed by immunofluorescence staining on GalT-KO.hCD55.hCD59.hCD39.hHT*Tg* kidney (1A) and by FACS on human (HAEC) and porcine aortic endothelial cells (PAEC) (1B): *first row*: GalT-KO.hCD55.hCD59.hCD39.hHT*Tg* PAEC (donor from Control group and groups #1–3), *second row*: GalT-KO PAEC (donor from group #4), *third row*: PAEC WT and *fourth row*:

HAEC. Histograms show negative control (cells without Ab, dashed line) and endothelial cells with Ab (plain line).

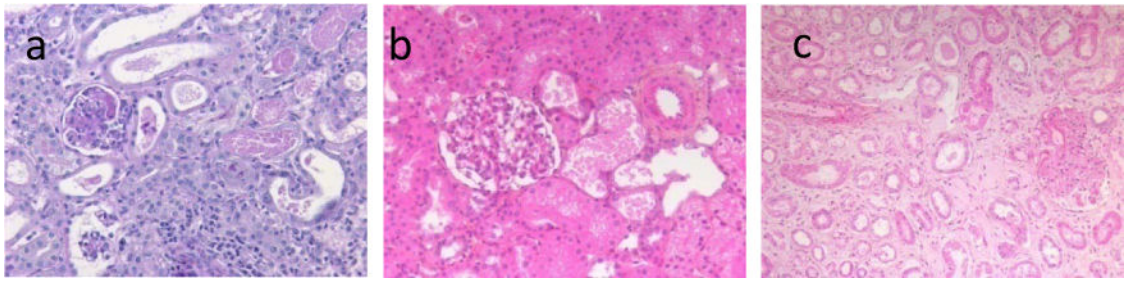


Figure 2.

Histological analyses of animals from control group (a: recipient #V857I) and from group#3 (recipient #K921F:b, c). a (×20), c (×10) showed kidney biopsies at rejection respectively at day 3 and d14 whereas b (×20) showed protocol biopsies at d4 in functioning graft.

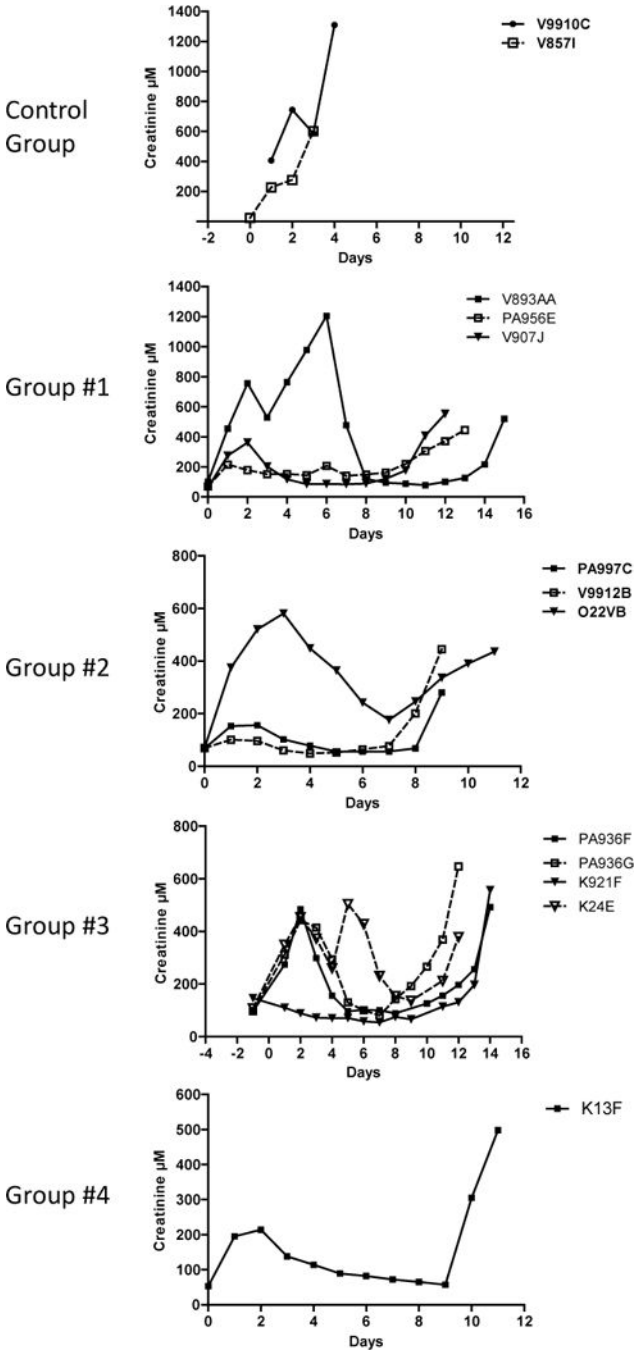
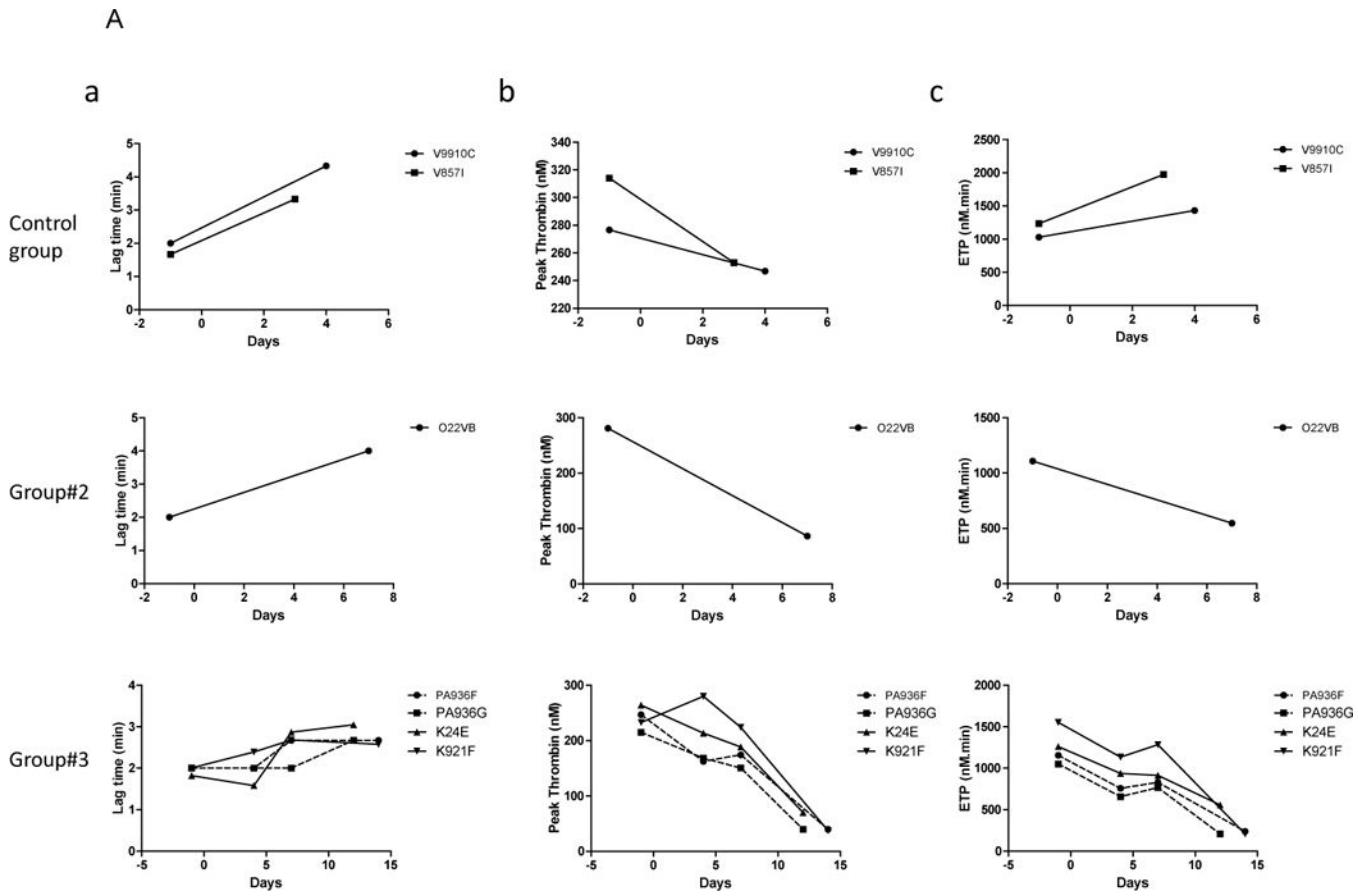


Figure 3.
Creatininemia of recipients was measured daily (µM).



B

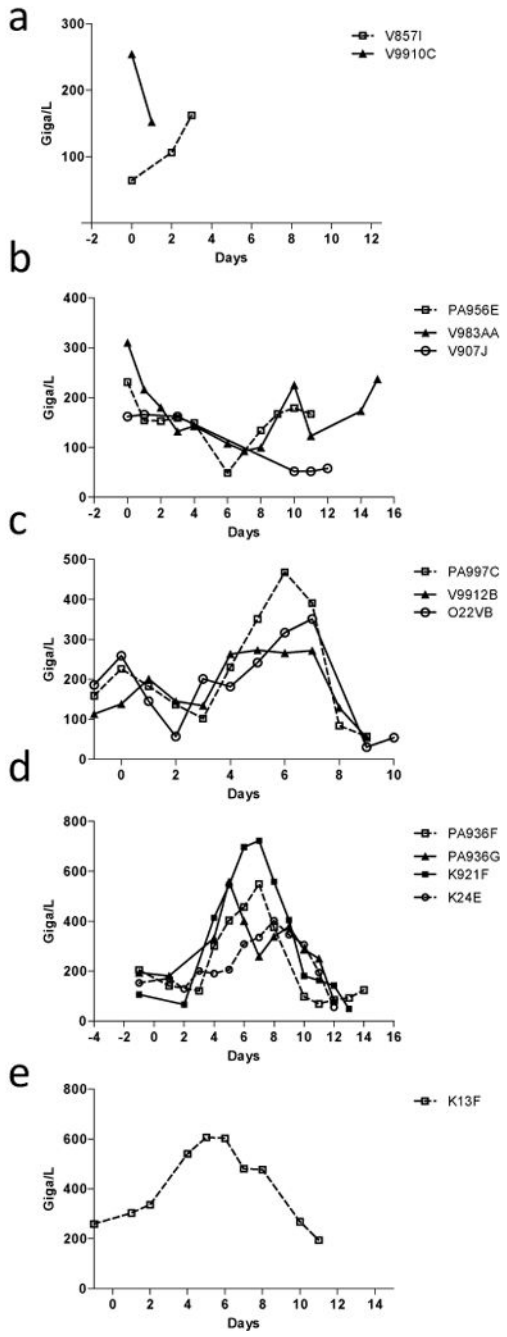
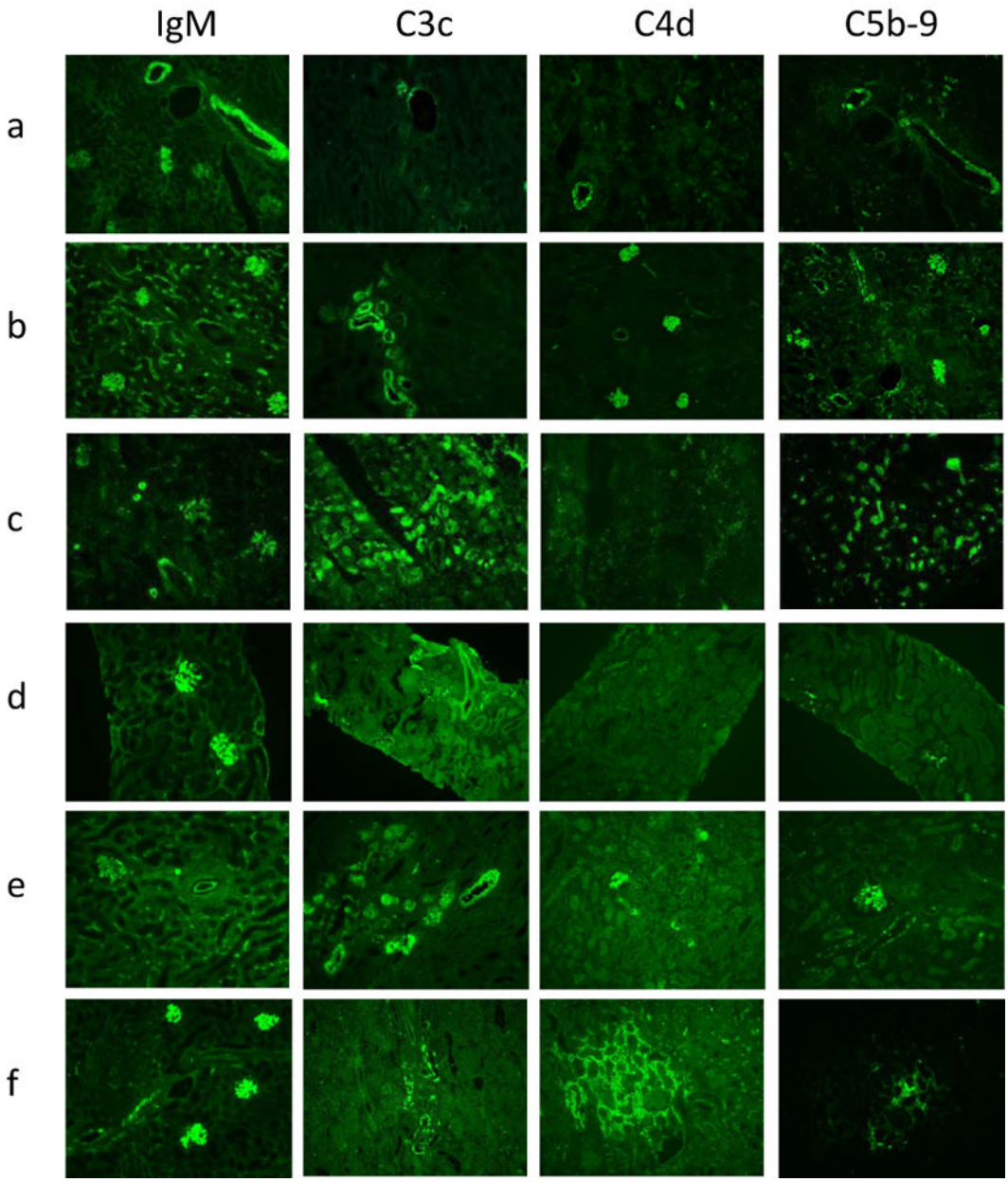


Figure 4.

Coagulation. Thrombin generation curves (4A) were calculated and evaluated by measuring (a) Lag time (min), (b) Peak thrombin (nM) and (c) area under the curve (ETP, nM.min) *first row:* in control group (recipients #V9910C and #V857I), *second row:* in group #2 (recipient #O22VB) and *third row:* in group #3 (recipients #PA936F, #PA936G, #K24E and #K921F). Prolongation of Lag-times and lower values of both peak thrombin and ETP are indexes of hypocoagulability. Platelets level was expressed in Giga/L (4B), in recipient from control group (a), group #1 (b), group #2 (c), group #3 (d) and group #4 (e).

A:



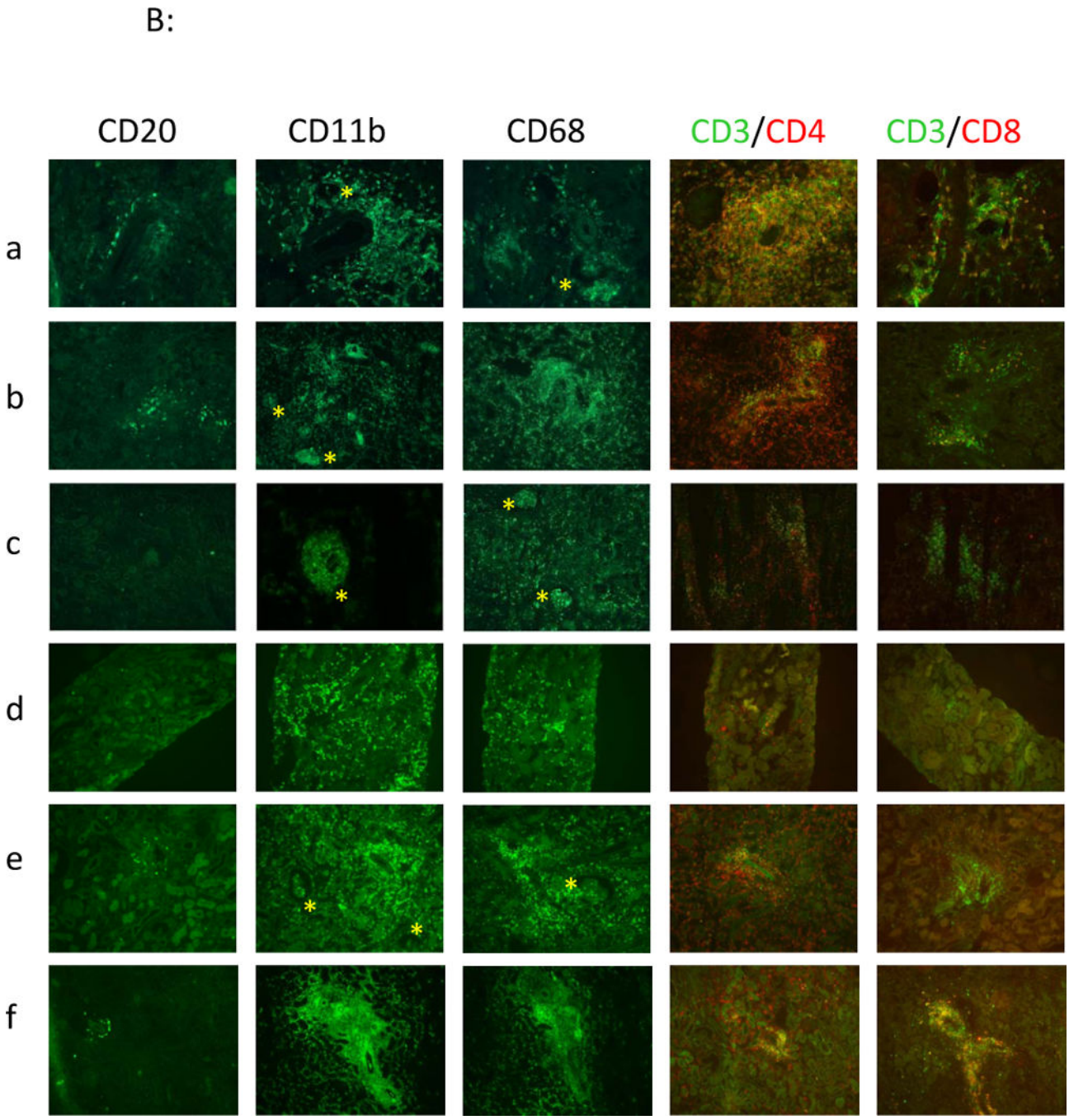


Figure 5.

Immuno-histofluorescence analyses. IgM, C3c, C4d, C5b-9 staining (4A) and cellular staining: B cells (CD20), monocytes/macrophages (CD11b, CD68): yellow star is emphasizing the presence of monocytes/macrophages in glomerular capillaries, CD4 and CD8 T cells (4B), were performed in frozen kidney biopsies from recipients a: control group (recipient #V9910C) at rejection at d3, b: from group#1 at rejection at d15 (recipient #V893AA), c: from group# 2 at rejection at d9 (recipient #PA997C) and d & e: from

group#3 (recipient #PA936G) respectively at d4 in functioning graft (d) and at rejection at d12 (e), and f: from group#4 (recipient #V906F) at rejection at d11.

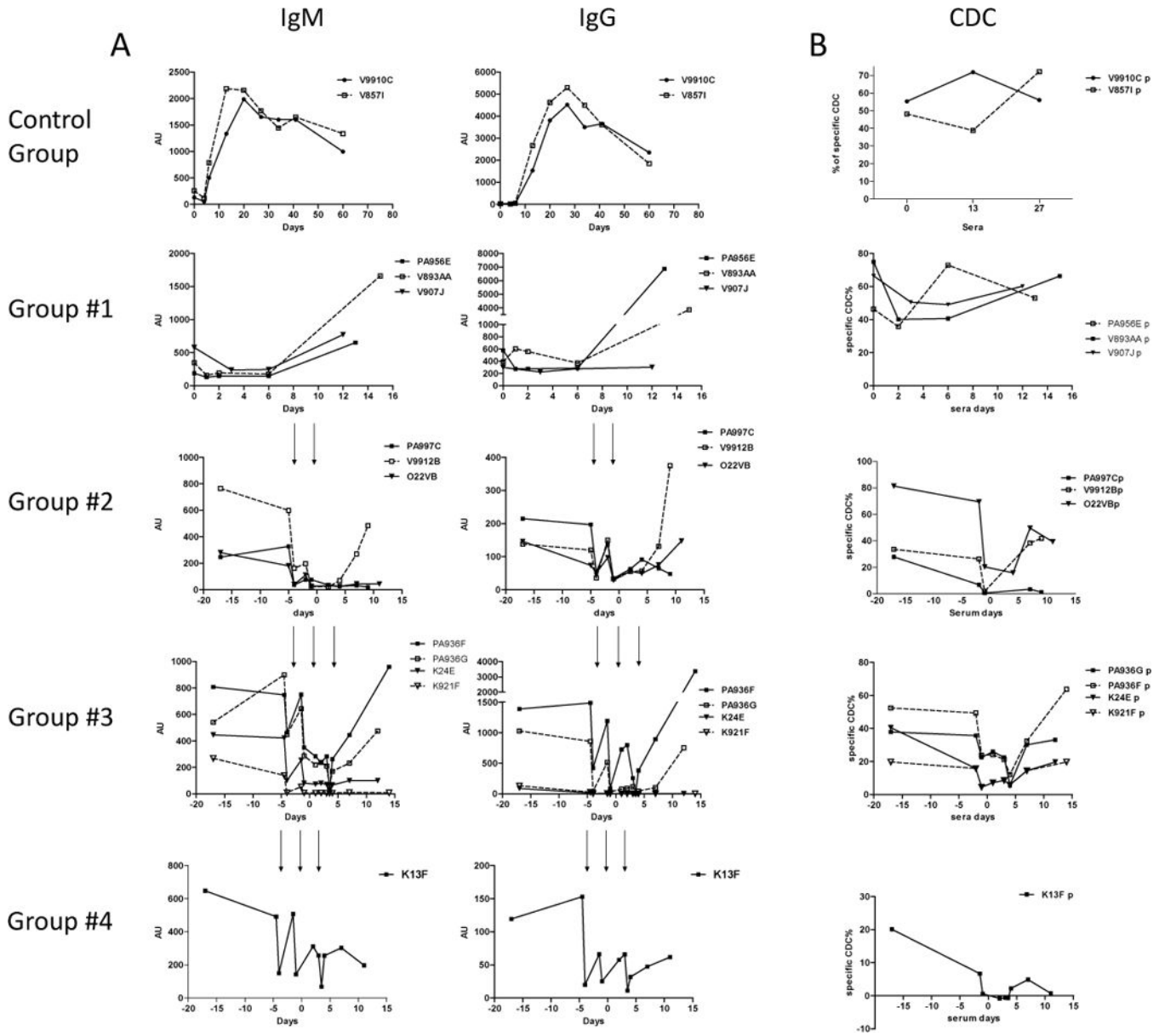


Figure 6.

Baboon serum xenoreactivity (6A) and cytotoxicity (6B) were assessed by FACS. A: IgM and IgG anti-nonGal antibodies were assessed by crossmatch using donor PAEC by FACS. Arrows show the decrease of circulating XNA after each PE. B: serum complement dependant cytotoxicity was assessed by a Flow cytometry Complement-mediated Cytotoxic Assay on donor PAEC.

Table 1

Summary of experimental conditions for the pig kidney to baboon study. Baboon recipient immunosuppression consisted of splenoctomy, plasma exchange and pharmacological treatment as outlined.

Groups	Recipients	Pig donors	Genetic modifications of donors	Splenectomy	Plasma exchanges	Pharmacological Treatment			Graft survival (days)	Diagnosis
Control group	V9910C	#007	GT-KO, tg hCD55, hCD59, hCD39, hHT	+	−		−		4	AXHR & AXCR
	V857I	#007	Id.	+	−		−		3	Id.
Group #1	V893AA	#005	GT-KO, tg hCD55, hCD59, hCD39, hHT	−	−	MMF, CS, Tacrolimus	Cyclophosphamid	C1INH 200U/ Kg d0 to d5	15	AXHR & AXCR
	PA956E	#005	Id.	−	−	Id.	Id.	Id.	13	Id.
	V907J	#006	Id.	−	−	Id.	Id.	Id.	12	Id.
Group #2	PA997C	#089	GT-KO, tg hCD55, hCD59, hCD39, hHT	+	**d-4, −1**	MMF, CS, Tacrolimus	**Bortezomib**	C1INH 200U/ Kg **d5 to d9**	9	AXHR & AXCR
	V9912B	#089	Id.	+	Id.	Id.	Id.	Id.	9	Id.
	022VB	#087	Id.	+	Id.	Id.	Id.	C1INH **400U/ Kg** d5 to d9	11	Id.
Group #3	PA936G	#095	GT-KO, tg hCD55, hCD59, hCD39, hHT	+	**d-4, −1, 3**	MMF, CS, Tacrolimus	Bortezomib	C1INH 400U/ Kg **d4 to d8**	12	AXHR & AXCR
	PA936F	#095	Id.	Id.	Id.	Id.	Id.	Id.	14	Id.
	K24E	#094	Id.	Id.	Id.	Id.	Id.	Id.	12	Id.
	K921F	#094	Id.	Id.	Id.	Id.	Id.	Id.	14	Id.
Group #4	K13F	#195	**GT-KO**	+	d-4, −1, 3	MMF, CS, Tacrolimus	Bortezomib	C1INH 400U/ Kg d4 to d8	11	AXHR & AXCR

GT-KO: Galactosyl-transferase knock-out tg: transgenic, HT: Fucosyl-transferase, MMF: Mycophenolate Mofetil, CS: corticosteroids, Id.: Idem, rhC1INH: recombinant human C1 Inhibitor, given every 8 hours, AHXR: Acute Humoral Xenogeneic Rejection, ACXR: Acute Cellular Xenogeneic Rejection.

Table 2

PERV analysis.

Groups	Recipients	PERV vRNA in serum (copies/ml)	PERV: centromeric ratio	Donor PERV: centromeric ratio
Group #1	V893AA	Neg	0.0188	0.032
	PA956E	Neg	0.0285	0.032
	V907J	$5.9*10^2$	0.0375	0.0604
Group #2	PA997C	$4.8*10^4$	0.0267†	0.0221
	V9912B	$2.7*10^4$	0.0148	0.0221
	O22VB	$3.4*10^4$	0.0144	0.0195
Group #3	PA936G	$4.5*10^5$	0.0884‡	0.0593
	PA936F	$9.9*10^5$	0.0182	0.0593
	K24E	$2.7*10^4$	0.0281	0.0285
	K921F	$1.3*10^4$	0.0238	0.0285
Group #4	K13F	$2.9*10^3$	0.0211	0.0236

† p=0.315

‡ p=0.227

Table 3

Summary of BCMV and PCMV copy number per 500ng DNA in donor graft and recipients.

Group	Graft Survival (days)	NHP ID	Pig donors	BCMV			PCMV			
				Recipient PBMC Pre-XT	Recipient PBMC Post-XT	Xenograft	Donor PBMC	Donor Spleen	Recipient Post-XT	Xenograft
Group #1	15	V893AA	#005	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	13	PA956E	#005	46 ± 15	270 ± 46	NEG	NEG	NEG	NEG	NEG
	12	V907J	#006	NEG	238 ± 8	NEG	NEG	NEG	NEG	NEG
Group #2	9	PA997C	#089	NEG	117 ± 5	NEG	7 ± 1	2 ± 1	NEG	153 ± 1
	9	V9912B	#089	NEG	NEG	NEG	7 ± 1	2 ± 1	27 ± 1	566 ± 7
	11	O22VB	#087	nd	Nd	NEG	NEG	9 ± 1	nd	571 ± 17
Group #3	12	PA936G	#095	NEG	NEG	NEG	5 ± 2	6 ± 1	370 ± 3	4469 ± 31
	14	PA936F	#095	NEG	NEG	NEG	5 ± 2	6 ± 1	41 ± 1	7646 ± 13
	12	K24E	#094	23 ± 4	79 ± 7	NEG	4 ± 2	9 ± 2	450 ± 16	47355 ± 3538
	14	K921F	#094	NEG	NEG	NEG	4 ± 2	9 ± 2	373 ± 2	92332 ± 803
Group #4	11	K13F	#195	253 ± 15	3194 ± 329	NEG	NEG	NEG	NEG	NEG

Table 4

Summary of experimental results according to recipients' groups.

Groups	Recipients	Graft survival (days)	Diagnosis	Immuno-histology at rejection		Peripheral anti-nonGal Ab		Virology		
				Humoral staining	Cellular infiltrate	Preformed at XT	Elicited at rejection	PERV: R/D centromeric ratio	Intragraft BCMV	Intragraft PCMV
Control group	V9910C	4	AXHR & AXCR	IgM, C3c, C4d, C5b-9	Monocytes/CD4+	IgM/IgG CDC	IgM/IgG CDC	ND	ND	ND
	V857I	3	Id.	Id.	Id.	Id.	Id.	ND	ND	ND
Group #1	V893AA	15	AXHR & AXCR	IgM, C3c, C4d, C5b-9	Monocytes/CD4+	IgM/IgG CDC	IgM/IgG CDC		Neg	Neg
	PA956E	13	Id.	Id.	Id.	Id.	Id	<1	Neg	Neg
	V907J	12	Id.	Id.	Id.	Id.	IgM CDC	<1	Neg	Neg
Group #2	PA997C	9	AXHR & AXCR	IgM, C3c, C4d, C5b-9	Monocytes/CD4+	IgM/IgG CDC	*	>1 (NS)	Neg	+/−
	V9912B	9	Id.	Id.	Id.	Id.	IgM/IgG CDC	<1	Neg	+
	022VB	11	Id.	Id.	Id.	Id.	IgG CDC	<1	Neg	+
Group #3	PA936G	12	AXHR & AXCR	IgM, C3c, C4d, C5b-9	Monocytes/CD4+	IgM/IgG CDC	IgM/IgG CDC	>1(NS)	Neg	++
	PA936F	14	Id.	Id.	Id.	Id.	Id.	<1	Neg	++
	K24E	12	Id.	Id.	Id.	Id.	*	<1	Neg	+++
	K921F	14	Id.	Id.	Id.	Id.	*	<1	Neg	+++
Group #4	K13F	11	AXHR & AXCR	IgM, C3c, C4d, C5b-9	Monocytes/CD4+	IgM/IgG CDC	*	<1	Neg	Neg

AHXR: Acute Humoral Xenogeneic Rejection, ACXR: Acute Cellular Xenogeneic Rejection, CDC: Complement Dependant Cytotoxicity

*elicited anti-nonGal Ab level and CDC at rejection < preformed anti-nonGal Ab level and CDC at XT, Neg: negative, ND: not done, NS: non significatnt. R/D: recipient/donor, XT: xenotransplantation.