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Development Of A Consensus Protocol To Quantify Primate Anti-Non-Gal Xenoreactive Antibodies Using Pig Aortic Endothelial Cells

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Abstract

Scientists working in the field of xenotransplantation do not employ a uniform method to measure and report natural and induced antibody responses to non-Gal α (1,3)Gal (non-Gal) epitopes. Such humoral responses are thought to be particularly pathogenic after transplantation of vascularized GalTKO pig organs and having a more uniform assay and reporting format would greatly facilitate comparisons between laboratories. Flow cytometry allows examination of antibody reactivity to intact antigens in their natural location and conformation on cell membranes. We have established a simple and reproducible flow cytometric assay to detect antibodies specific for non-Gal pig antigens by using primary porcine aortic endothelial cells (pAECs) and cell culture adapted pAEC cell lines generated from wild type and α 1,3galactosyl transferase knockout (GalTKO) swine. The consensus protocol we propose here is based on procedures routinely used in four xenotransplantation centers, and was independently evaluated at three sites using shared cells and serum samples. Our observation support use of the cell culture adapted GalTKO pAEC KO:15502 cells as a routine method to determine the reactivity of anti-non-Gal antibodies in human and

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baboon serum. In conclusion, we have developed an assay that allows the detection of natural and induced non-Gal xenoreactive antibodies present in human or baboon serum in a reliable and consistent manner. This consensus assay and format for reporting the data should be accessible to most laboratories and will be useful for assessing experimental results between multiple research centers. Adopting this assay and format for reporting the data should facilitate the detection, monitoring, and detailed characterization of non-Gal antibody responses.

Keywords

Antibody; non-Gal; antibody; xenoreactive; assay; galactosyl transferase; cell line; xenotransplantation

Introduction

The inadequate supply of human organs for transplantation is a major limitation to comprehensive and widespread therapy of end-stage organ failure. Xenotransplantation has the potential to solve the problem of organ supply, as it would make organs available whenever needed [1,2]. Pigs are considered to be the most promising source animals for clinical use due to their size, physiology, breeding characteristics, and low risk for transmitting infection to man [3-5]. However, transplantation of vascularized xenografts into humans results in hyperacute rejection due to the presence of preformed anti-pig antibodies which bind the major xenogeneic antigen Gal α (1,3)Gal (Gal) resulting in subsequent activation of the complement and coagulation cascades [6-9]. Previous studies have used synthetic alpha-galactosyl glycoconjugates, and removal of anti-pig antibodies by absorption to neutralize antibodies specific for the Gal epitope [10-13].

The recent development of pigs in which the α -galactosyl transferase gene has been deleted (GalTKO) [14-17] and human complement regulatory proteins have been over-expressed [13,18-20] has further reduced [21,22] but not eliminated the incidence of hyperacute rejection [19,23]. Xenotransplantation using GalTKO organs has eliminated a role for anti-Gal antibody in xenograft rejection but has not eliminated antibody mediated rejection overall as both preformed and induced antibodies binding to GalTKO organs (i.e. antibody that binds to GalTKO pig endothelial cells) contribute to xenograft rejection. Several groups are evaluating strategies to develop clinically-applicable immunomodulatory regimens to prevent these residual immune responses [24-28]. As part of this strategy it is essential to identify the presence of potentially pathogenic preformed xenoreactive antibodies against non-Gal pig antigens, and monitor their de novo induction.

In an effort catalyzed by the NIH Xenotransplantation Consortium, four laboratories collaborated on the development and evaluation of a broadly accessible protocol to measure anti-pig antibodies, with the goal of developing consensus on existing experimental procedures and reporting formats [22,25,29-31]. The resulting assay described in this report provides a sensitive and reproducible method to measure both natural and induced human and non-human primate antibodies reacting with non-Gal pig antigens using GalTKO endothelial cells and flow cytometry. This consensus assay and the format in which the data

is reported should be easily accessible to most laboratories and adoption of this method will facilitate the comparison of non-Gal antibody responses between multiple research centers.

Materials and Methods

Cell isolation and culture

Primary porcine aortic endothelial cells (pAECs) were isolated by collagenase digestion and/or mechanical scrapping as previously described [32-34] and seeded in gelatin-coated culture flasks (Sigma #G1393, 0.5%). Primary cells must be monitored for their quality (morphology, CD31 expression) and used in early passages (up to passage 6 generally). Tissue culture adapted wild-type (WT:14259) or GalTKO (KO:15502) pAEC lines from miniature swine were kindly provided by Dr. Hendrik-Jan Schuurman, formerly of Immerge BioTherapeutics. Cell lines will be made available to other centers upon request. All cells were cultured in Dulbecco's modified Eagles medium (DMEM) containing 1,000 mg/L D-glucose, 110 mg/L sodium pyruvate, L-glutamine or glutamax, antibiotics, 10% heat-inactivated fetal calf serum and 50 µg/ml endothelial cell growth supplement (ECGS, BD, Ref 356006). ECGS was used at 100 µg/ml for the first passage of primary pAECs.

Flow cytometry staining for αGal and CD31

Expression of αGal residues was detected by staining with the lectin *Bandeiraea simplicifolia* Isolectin B4 (BS-I-B₄) (Sigma # L-2895, 1 µl/1×10⁶ cells). The endothelial cell phenotype was assessed by staining for expression of CD31 using FITC-labeled mouse-anti-pig CD31 antibody (Serotec # MCA 1746F, 1:10) or FITC-labeled isotype control.

Serum samples

All samples were heat-inactivated prior to use as indicated in Table 1. A naïve human serum pool was used as internal control in all experiments (Sigma, Ref. H4522). A naïve baboon serum pool was prepared by mixing equal amounts of serum from ten naïve non-transplanted baboons. Additional serum samples (BS1 - 6) were from baboons sensitized in association with rejection of GalTKO pig heart xenografts [33-36]. Shared serum samples were heat-inactivated in the source center, and frozen aliquots were shipped to the other two evaluation centers. Serum dilution volumes were standardized between the three centers. All baboons used in this study were apparently healthy and free of disease as defined by screening procedures in place at each Institution.

Staining procedure

The staining procedure technical details were resolved among four independent research teams, based on iterative pilot studies evaluating various proposed culture media, secondary antibodies and staining conditions. The choice of secondary anti-human antibodies was investigated by direct comparison of different commercial reagents. We found that non-human primate (NHP)-specific reagents are not widely available, and those that are available cannot also be used to assay human anti-pig responses. Secondary antibodies were chosen based on their cross-reactivity in baboon, availability and sensitivity in pilot studies (data not shown): PE- goat anti-human IgM Fc (Southern Biotech #2020-09) and FITC- goat anti-

human IgG Fc (Invitrogen #62-8411). The consensus protocol used for cell culture and collection conditions, cell staining and analysis is described in Table 1.

Flow cytometry equipment

The standardized protocol was evaluated by three independent laboratories respectively equipped with a FACs Calibur (Laboratories #1 and #2) and an LSRII (Laboratory #3). A few experiments were also performed using a FACs Verse in Laboratory #1. All flow cytometers were from Becton Dickinson (BD, San Jose, CA), and all laboratories have extensive contemporary experience with the culture of pAECs and with FACs.

Statistical analysis

Data are presented as mean $\pm$ standard deviation for all parametric variables. Correlations between parameters were assessed using the Pearson correlation test. Values of p less than 0.05 were considered statistically significant. Assay variability was expressed as the quartile coefficient of dispersion (CD) calculated as the interquartile range ($Q_3 - Q_1$) divided by the median (Q_2). Statistical analyses were performed with Excel or with the statistical package GraphPad InStat (La Jolla, CA) for Windows (version 3.01).

Results

Phenotypic characterization of pAEC lines from WT and GalTKO pigs

The culture adapted cell lines WT:14259 and KO:15502 are easily cultured and a convenient cell for detecting Gal and non-Gal antibody reactivity in primate sera. The intensity of staining for Gal on WT:14259 cells matches primary porcine aortic endothelial cells and the KO:15502 cells do not express the Gal antigen (Fig. 1A). Moreover, the culture adapted cells appear to be a homogenous population with uniform expression of CD31. Primary PAECs can be contaminated with CD31 negative fibroblasts when isolated and are frequently overgrown by these cells on extended passage (Fig. 1B). The GalTKO pAEC line (KO:15502) was chosen as a consistent cell source to develop and evaluate a consensus protocol to detect non-Gal antigens.

Development and reproducibility of a consensus flow cytometry protocol

A consensus flow cytometry protocol was established (see Methods and Table 1) after extensive comparison of flow cytometry conditions and optimization of secondary reagents and experimental conditions used within our four research groups. This protocol was used to compare antibody binding on WT:14259 and KO:15502 cell lines. As expected, antibody binding was higher on WT pAECs (expressing both Gal and non-Gal epitopes) compared to GalTKO pAECs (expressing non-Gal epitopes only), particularly for human and baboon IgM reactivity (Fig. 2). Non-Gal IgG reactivity to KO:15502 cells showed a bell-shaped curve characteristic of a prozone artifact [37].

Four serum samples were analyzed in parallel by three laboratories (designated as Lab#1 - 3) in order to measure variability between different sites in the assay. Each site used the consensus protocol, to test four samples: a naïve human serum pool, a naïve baboon serum pool and serum samples from two baboons that received a GalKO heart xenograft purposely

performed in absence of effective immunosuppression (BS1 and BS2). Results from the three centers are represented as median fluorescence intensity (MFI) in Fig. 3. As expected, non-Gal antibody reactivity was higher for BS1 and BS2 baboon sera (blue and green) compared to naïve serum pools (orange and red), a finding common to all three centers. We found that the assay was reproducible within each site (small error bars) and generally results for non-Gal IgM reactivity were qualitatively similar between centers (Fig. 3A). Specifically, each center identified BS2 with the highest non-gal IgM reactivity, followed by BS1 and naïve pools (blue>green>pink>red). Non-Gal IgM reactivity uniformly showed a continuous decrease in MFI with serum dilution (range 1:4 to 1:1024) at each center (Lab#1 - 3). In contrast, more variable results for IgG binding to GalTKO cells were observed between centers, particularly for the sensitized (BS1 and BS2) baboon samples (Fig. 3B). At two sites BS2 showed the highest non-Gal IgG reactivity (blue in Lab#1 and Lab#2), but at the third site the highest reactivity was observed for BS1 (green in Lab#3, Fig. 3B, top panel). The non-Gal IgG dilution profile showed a bell-shaped distribution with maximum fluorescence intensity at or near the 1:64 serum dilution in two centers (Fig. 3B, Lab#1 and #3). In an effort to reduce inter-center variability, the non-Gal IgG results were expressed in relative fluorescence units (RFU) by standardizing the fluorescence to either the no serum control (Fig. 3B, middle panel) or the human naïve pool (Fig. 3B, bottom panel). This standardization resulted in a modest reduction in relative reactivity (RFU) for BS1 in Lab#3 but did not substantially affect the results in Lab#1 and 2 (Fig. 3B).

Intra-assay variability was tested for non-Gal IgM reactivity by analysis of technical replicates (triplicate tubes) for naïve human serum in one experiment at two centers. Intra-assay variability, estimated by quartile coefficient of dispersion (CD), was consistently low ranging from 1.4 % to 7% at both centers. Next, inter-assay variability was calculated for four serum samples tested in three experiments at each individual site. Inter-assay variability was higher than intra-assay variability but remained relatively low in all study sites (generally below 30%) for both non-Gal IgM and IgG (Table 2). Inter-assay variability was also studied over a period of two years at one of the centers for human naïve reactivity, and showed more variability for IgM (55%) than for IgG (16%). Finally, the average result of the three independent experiments performed at each center was used to calculate the assay variability between centers. As discussed above for Figure 3, inter-center MFI variability was highest for IgG and for sensitized baboon samples (BS1 and 2), ranging from 13% to 38% for IgM, and from 11% to 63% for IgG (Table 3). The standardization of units was associated with lower inter-center variability when RFU were calculated using the no serum control (CD: 37) but not using the human pool (CD: 46), compared to MFI units (CD: 45) (Table 3, IgG 1:64), whereas the lowest variability for IgM naïve reactivity was observed with RFU calculated using the human pool (Table 3, IgM 1:4).

The sources of inter-assay and inter-center variability were further evaluated by examining the effects of both cytometer calibration and cell culture conditions on antibody reactivity. We compared results using two different FACs instruments. The MFI comparison between instruments showed differences in the scale of signal detection with a higher sensitivity observed with the digital (FACs Verse) compared to the analog (FACs Calibur) flow cytometer (Fig. 4A, MFI scale). Despite this apparent difference in sensitivity the results for both instruments were highly correlated (Fig. 4; IgM $R^2=0.93$ and IgG $R^2=0.81$). The

correlation of antibody reactivity between instruments was improved when MFI results were standardized using the RFU calculation ($R^2=0.98$ and 0.99 respectively) (Fig. 4A).

We also found that cell culture conditions affect the reactivity of non-Gal antibody. To assess the effect of cell confluence on antibody reactivity, cells were seeded at various densities to achieve incomplete (<50%), near-confluent (80-100%) and confluent (>100%) cell monolayers. Both IgM and IgG reactivity were maximal using almost confluent (80-100%) cells. This was most evident for IgG binding from sensitized baboon serum (BS3) where antibody binding to almost confluent cells was increased more than 2-fold compared to the other cell culture conditions (Fig. 4B).

Relevance of the GalTKO cell line compared to primary cells

To determine whether the KO:15502 pAEC cell line expresses antigens shared by different individual pigs and thus represents a generally suitable antigenic target cell line, we compared antibody binding to KO:15502 and primary pAECs. Similar amounts of naive human IgM and IgG bound to the KO:15502 cell line and to 3 different primary pAEC cultures (Figure 5A). We also examined antibody reactivity to the KO:15502 cell line, donor-type pAECs and third-party primary pAECs in serum samples from three baboons previously transplanted with a GalKO pig xenograft under immunosuppression (BS4 - 6, see Table 4). Antibody reactivity to KO:15502 was often similar (as illustrated for baboons BS4 and BS5) to donor-type and to third-party primary pAECs (Fig. 5B). For some serum samples however, reactivity to the KO:15502 GalTKO cell line was reduced compared to donor (Baboon BS6, red vs. blue), or third party (Baboon BS6, red vs. black) primary pAECs.

Discussion

The development of pigs lacking the α Gal antigen has eliminated the role of anti-Gal antibody in xenograft rejection but not fully blocked antibody-mediated delayed xenograft rejection. Several groups have reported the presence and/or induction of anti-pig antibodies in response to GalTKO organ transplantation. These non-Gal antibodies are frequently present in nonhuman primates at the time of transplant [19,21,28,29,31,38], increase after transplant, and are strongly associated with organ rejection. Non-Gal antibody reactivity has variously been reported based on assays that measure antibody binding to porcine peripheral blood mononuclear cells, red blood cells and aortic endothelial cells [22,25,29-31]. Results have been reported at a single serum dilution or based on a dilution profile, and the values have been reported as raw data or in various normalized formats. This heterogeneity makes it difficult to compare results between transplant centers. To address this issue, a collaborative effort was initiated within the members of an NIH consortium to develop and evaluate a standardized assay to monitor non-Gal xenoreactive antibodies. Here we present a consensus protocol that, if adopted, we believe will facilitate more dependable comparison, and thus more informative interpretation of xenoreactive antibody results between investigators, and between different experimental (and subsequent clinical) studies.

Unlike Gal-mediated xenograft rejection, no single non-Gal antigen has been identified which dominates rejection of GalTKO organs. Several porcine endothelial cell proteins have

been shown to be recognized by human natural antibody or by non-human primate serum after cardiac xenotransplantation [33,39]. There are also additional carbohydrate antigens, other than Gal, which are known or have been shown to induce antibody responses in human and non-human primates [36,40-45]. The contribution of antibody reactivity to these individual antigens (protein and carbohydrate) in xenograft rejection remains uncertain.

In the absence of a clearly defined non-Gal antigenic target we have chosen to use whole GalTKO endothelial cells as a source of pig antigens. The choice of endothelial cells was guided by the pathology of xenograft rejection which primarily is directed at vascular endothelial cells. Although peripheral blood cells are abundant, easy to isolate, and were historically used to monitor antibody sensitivity of allotransplant patients, these cells are not necessarily optimal in xenotransplantation. Evidence to date suggests that antibody mediated xenograft rejection involves a polyclonal pan pig reactivity which is largely not donor specific. These considerations suggested to us that pAECs represent the most appropriate target cell to quantify preformed and elicited anti-pig antibody.

In the course of our studies, we evaluated the use of primary pAECs and two novel cell culture adapted cell lines (WT:14259 and KO:15502). We show that antibody binding to these cell lines generally is in agreement with prior work comparing the reactivity of WT and GalTKO cells [46,47]. We also show that antibody reactivity to primary donor specific, third party and the culture adapted cell lines generally show a high level of overall concordance. This is consistent with the (pan-pig, polyclonal/polyreactive) nature of non-Gal antibody and indicates that cultured third-party primary pAECs or the KO:15502 cell line appear to be a convenient proxy to detect sensitized responses to non-Gal antigens.

Occasionally we found a much higher reactivity against donor and third party primary cells than against the KO:15502 cell line that we propose should be used for screening (Figure 5B, sample B3). These antibodies might react with donor-type swine leukocyte antigens (SLA) determinants not expressed by the cell line. There is some evidence for specific SLA haplotype reactivity among anti-non-Gal antibodies in the literature [48] although this immune specificity is not universal in xenotransplantation [49]. Alternatively, the cultured cell line might have lost expression of certain determinants expressed by primary cells. In addition, several human transgenes are now expressed by pig organs used for xenotransplantation. These human gene products may be immunogenic after pig-to-primate xenotransplantation. In some instances expression of human transgenes could potentially alter the expression of porcine antigens recognized by anti-non-Gal antibodies. These changes would only be present on donor specific pAECs and not on the KO:15502 cell line. Thus, while the culture adapted cell line is a useful proxy to detect preformed or elicited antibody against non-polymorphic pig non-Gal antigens and to compare data between research centers, primary pAECs derived from the donor pig strain remain the “gold standard” for detecting the entire repertoire of induced anti-donor antibodies in a particular experiment.

Cell-based assays for detecting antibody reactivity are typically based on three techniques: ELISA, complement-dependent cytotoxicity (CDC), and flow cytometry. ELISA techniques have previously been used to detect xenoreactive antibodies against Gal-expressing

endothelial cells [50,51]. This technique is labor-intensive, and the fixation process may modify protein epitopes, disrupt carbohydrate secondary structures [52], as well as expose intracellular antigens which may not contribute to xenograft rejection. The CDC and flow cytometry approaches use unfixed cells and thus have the advantage of expressing native, non-denatured, cellular antigens. The CDC assay only detects complement-fixing antibodies, whereas the flow cytometry technique allows the detection of all antibody subclasses, and is typically more sensitive to relatively subtle changes in antibody titer. These considerations justified our choice of the flow cytometry platform to develop a consensus method.

Our analysis shows that the flow cytometry-based anti-non-Gal consensus assay is sensitive to specific experimental conditions, primarily the cell culture conditions (Fig. 4) and cytometer related effects. In our experience, cells collected just prior to confluency are associated with the highest level of non-Gal antibody reactivity. This suggests that adoption of consistent target cell culture conditions will be an aid to interpreting results from different laboratories. Independent of target cell culture conditions, non-Gal IgG binding showed a bell shaped profile in many instances. The reason for this finding is not clear, but suggests that assaying and reporting serial dilutions is required to accurately assess non-Gal IgG binding and to measure (or exclude the presence of) prozone effects. Moreover, serum from baboons after cardiac xenotransplantation sometimes showed evidence of toxicity to the target cells manifest by a much increased time of acquisition (data not shown). This phenomenon occurs despite heat inactivation, but disappears with serum dilution, again supporting the importance of serial dilution to accurately measure an elicited or high-titer non-Gal antibody response. Different flow cytometer models have various physical properties that affect assay results, and which are determined by laser physics, laser power, and filters. For that reason different approaches have been proposed to standardize flow cytometry results between laboratories [53,54]. Specific protocols were recently developed using calibration beads such as the Becton Dickinson's Cytometer Setup and Tracking beads to create and save optimal instrument settings for specific applications and obtain consistent and reproducible results over time [55]. These beads could be used in the future to standardize multiple cytometers with different optical components across centers. We found that expression of results as relative fluorescence units standardized to the secondary only negative control limits some of the inevitable variability between centers. Using a GalTKO cell line and serum standards, the assay was noteworthy for being reproducible between laboratories. The use of relative fluorescence units further aided comparisons between laboratories, particularly for IgG.

One important caveat to this assay is that it is not designed to be sensitive for detection of anti-Neu5Gc antibody reactivity. Humans, but not nonhuman primates, produce antibodies which react with terminal Neu5Gc modified glycans. Unfortunately, the Neu5Gc antigen is ubiquitous in common cell culture reagents and thus the assay and culture conditions we describe would not be suitable for detecting anti-Neu5Gc antibody reactivity [56]. Specialized culture conditions, using Neu5Gc-free media, and staining reagents would be required to assess anti-Neu5Gc reactivity in human serum; recent establishment of a GalTKO. Neu5Gc-KO pAEC cell line [17] and Neu5Gc-specific assays [57] have been advanced to address this issue. Finally, it remains to be shown whether antibody binding to

pAECs by flow cytometry accurately predicts or correlates with antibody binding to the graft tissue.

In summary, through a collaborative consortium effort, we have developed a consensus protocol to detect antibodies against non-Gal pig antigens to facilitate inter-laboratory comparisons, and evaluated this approach using sera from baboons sensitized by GalKO cardiac xenografts. Whether the assay is suitable for xenotransplantation of other organs or pancreatic islets is not clear at this time, albeit our experience with kidney xenografts in baboons (Azimzadeh et al., unpublished) and islet xenografts in rhesus macaques [28] suggests that the assay is informative in those contexts as well. We offer recommendations on procedures and reagents and discuss sources of assay variability that should aid in the reporting of humoral immune responses to non-Gal epitopes. While methods for detecting non-Gal antibody are likely to evolve as new insights on the relevant antigens are made and new technologies emerge [33,36,39,41,42,58], this study provides an easily assessable method that we expect will facilitate comparisons between data reported by different research groups and that we hope will thus contribute to progress in the field of xenotransplantation.

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List of abbreviations in alphabetical order

BSA	Bovine serum albumin
CDC	Complement-dependent cytotoxicity
DMEM	Dulbecco's modified Eagle medium
ELISA	Enzyme-linked immunosorbent assay
FACs	Fluorescence-activated cell sorting
FSC	Forward scatter
Gal	Gal α (1-3)Gal
GalTKO	Galactosyl transferase knock-out
MFI	Median fluorescence intensity
pAEC	Porcine aortic endothelial cell
RFU	Relative fluorescence units
SSC	Side scatter
SB	Staining buffer

WT wild-type

Figure 1. Phenotypic characterization of WT and GalTKO porcine endothelial cell lines (KO: 15502 and WT:14259)

A. Expression of α Gal was assessed using FITC-conjugated lectin BS-I-B₄. B. Staining for CD31. Unstained cells were used as negative control for IB4 lectin staining, and an isotype-matched control was used for CD31 staining (grey).

Figure 2. IgM and IgG antibody binding to WT and GalTKO pAEC cell lines
 Serial dilutions (1:4 to 1:1024) of naïve human and baboon serum pools were tested using the standardized protocol described in the Methods section and Table 1. Data were expressed as median fluorescence intensity (MFI).

Figure 3. Inter-laboratory evaluation of the consensus protocol

The figure compared IgM (A) and IgG (B) reactivity for naïve human and baboon serum pools and serum from baboons sensitized by cardiac xenotransplantation (BS1 and BS2) tested using the pAEC line KO:15502 cells as measured in the three test sites. In panel B, median fluorescence intensity (MFI) units (top panel) were converted to relative fluorescence units (RFU) as a ratio of MFI to the secondary only control (RFU no serum, middle panel) or to the human pool (RFU hu pool, bottom panel), using the mathematical equations listed at the bottom. BS1, BS2: serum samples collected from baboons sensitized to pig antigens by a GalKO heart xenograft in absence of immunosuppression as indicated in Table 4.

Figure 4. Sources of variability

A, Comparison of results produced using different flow cytometers (FACS Calibur or FACS Verse), using MFI or RFU data analysis. B, Compares the effects of cell culture conditions on MFI values for IgM (left) or IgG (right) antibody reactivity. BS3: Serum sample collected from a baboon sensitized to pig antigens by a GalTKO heart xenograft in absence of immunosuppression as indicated in Table 4.

Figure 5. Comparison of antibody reactivity to primary and cell culture adapted GalTKO cell lines

A, The reactivity of naïve human serum was tested using the pAEC line KO:15502 (red) or primary pAECs from 3 individual GalTKO pigs (black lines); B, Serum samples from baboons sensitized by cardiac xenotransplantation (BS4 - 6) were tested using the pAEC line KO:15502 (red), primary pAECs from the donor pig (blue), or primary pAECs from unrelated third-party pigs (black). Since results were expressed as MFI or RFU, legend for the Y axis was omitted for clarity and units of measure are indicated on the top of each figure panel. The results from three representative animals are shown. BS4 - 6: Serum samples collected from baboons sensitized to pig antigens by a GalTKO heart xenograft with immunosuppression as indicated in Table 4.

Table 1
Outline of the consensus protocol

Step	Procedure
Cell preparation	<ul style="list-style-type: none"> - Expand cells in culture medium (0.5×10^6 cells/T75 flask). At 80- 95% confluency, trypsinize and expand (1×10^6 cells/T75 flask). - At 80-95% confluency, harvest cultured pAECs and adjust cell concentration to 2×10^6 cells/ml in staining buffer
Staining buffer (SB)	<ul style="list-style-type: none"> - PBS+1% BSA (Sigma #A7030), prepared fresh
Serum	<ul style="list-style-type: none"> - Heat-inactivate serum at 56°C for 30 min. Centrifuge at 13,000g for 5 min to pellet aggregates. Store at 4°C for up to one week until use. - On the day of the assay, prepare serum dilutions in SB
Primary stain	<ul style="list-style-type: none"> - Mix 50 μl of cell suspension with 50 μl of appropriate serum dilution in FACs tubes (BD Falcon #352052). - Incubate at 4°C for 45 min.
Wash	<ul style="list-style-type: none"> - Add 2 ml of cold SB, centrifuge at $\sim 350\text{g}$, decant supernatant
Secondary stain	<ul style="list-style-type: none"> - Add 100 μl of secondary antibody diluted in cold SB to the cell pellet: anti-human IgM (Southern Biotech) diluted 1:50 or anti-human IgG (Zymed) diluted 1:20. - Incubate at 4°C for 30 min
Wash and resuspend	<ul style="list-style-type: none"> - Add 2 ml of cold SB, centrifuge at $\sim 350\text{g}$, decant supernatant, resuspend cells in 300 μl of PBS.
FACs acquisition	<ul style="list-style-type: none"> - Adjust voltage values for FSC and SSC to visualize the entire cell population. - Adjust the voltage for the PE channel so that the MFI of the negative control (no serum) is ~ 3. Acquire 10,000 gated events.
Data analysis	<ul style="list-style-type: none"> - Gate the cell population, excluding dead cells and debris. - Calculate the median fluorescence intensity (MFI) for each tube.

Table 2

Assessment of inter-assay variability at different centers

	IgM						IgG					
	Lab#1		Lab#2		Lab#3		Lab#1		Lab#2		Lab#3	
	Median	CD	Median	CD	Median	CD	Median	CD	Median	CD	Median	CD
Human naïve pool	46	19	57	2.4	24	7.4	20	23	9.8	23	11	8.8
Baboon naïve pool	47	23	68	15	31	21	17	9.4	7	30	10	13
BS1	115	39	129	14	57	28	48	22	25	10	63	32
BS2	437	30	367	22	347	14	64	15	40	4	69	29

Four serum samples were tested in three independent experiments at each center. Data are shown for serum dilution 1:4 (IgM) and 1:64 (IgG), except for BS2 which was analyzed at 1:16 for IgM. Data were expressed as the median and quartile coefficient of dispersion (CD) of the MFI values from individual experiments.

Table 3
Assessment of inter-center assay variability

Ab	Unit		Human naïve pool	Baboon naïve pool	BS1	BS2
IgM (1:4)	MFI	Median	42	47	106	391
		CD	38	35	31	13
	RFU (no serum)	Median	11	13	26	93
		CD	38	26	38	23
	RFU (hu pool)	Median	1	1.2	2.4	7.9
		CD	0	1.2	5	52
IgM (1:64)	MFI	Median	8.8	9.5	13	130
		CD	22	25	21	13
	RFU (no serum)	Median	2.1	2.3	3.1	33
		CD	39	43	38	33
	RFU (hu pool)	Median	0.8	0.2	0.3	3
		CD	20	25	21	46
IgG (1:4)	MFI	Median	8.2	6.0	48	62
		CD	11	29	63	33
	RFU (no serum)	Median	1.8	1.8	12.7	15.2
		CD	15	20	47	17
	RFU (hu pool)	Median	1	0.8	6	7.8
		CD	0	17	46	33
IgG (1:64)	MFI	Median	11	11	47	58
		CD	54	49	45	27
	RFU (no serum)	Median	2.4	2.6	13.3	15.3
		CD	102	79	37	36
	RFU (hu pool)	Median	1.6	1.5	6.4	7.9
		CD	54	53	46	30

Four serum samples were tested in three independent experiments at each center. Data are shown for serum dilution 1:4 and 1:64, except for BS2 which was analyzed at 1:16 for IgM. Data were expressed as the median and quartile coefficient of dispersion (CD) between the averages of three independent experiments performed at each center.

Table 4
Summary of baboon samples used in this study

Sample ID ^I	Center	Clinical history	Graft survival time	Time post-transplant
Naïve pool	Lab#3	Ten naïve non-SPF baboons	N/A	N/A
BS1	Lab#3	Heterotopic GalTKO heart,	2 hours ^a	120 days
BS2	Lab#3	no immunosuppression	8 days ^b	21 days
BS3	Lab#1		1 day	15 days
BS4	Lab#1	Heterotopic GalTKO heart,	7 days	14 days
BS5	Lab#1	T cell depletion, CD154	7 days	14 days
BS6	Lab#1	costimulation blockade	10 days	26 days

^I Baboon serum samples used in this study. Naïve pool represents an aggregate of baboon serum from individuals (n=10) with no known prior exposure to pig tissue. BS1 - 6 are baboon serum after cardiac xenotransplantation. BS1 - 3 are post-explant serum samples from pig-to-baboon cardiac xenotransplantations performed in the absence of immunosuppression. They have very high levels of non-Gal antibody. BS4 - 6 are post-transplant serum samples with more moderate levels of induced non-Gal antibody.

^a This xenograft recipient became hemodynamically unstable and the graft was explanted after 2 hours. Remnants of pig aorta and pulmonary artery remained in the baboon after excision.

^b Details about this baboon were published previously [36]. N/A not applicable