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ORIGINAL ARTICLE

Incidence and cytotoxicity of antibodies in cynomolgus monkeys directed to nonGal antigens, and their relevance for experimental models

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Summary

The recent availability of pigs homozygous for α1,3-galactosyltransferase gene-knockout (GT-KO) has enabled the study of incidence and cytotoxicity of antibodies of cynomolgus monkeys directed to antigens other than Galα1,3Gal (Gal), termed nonGal antigens. To this aim, sera from 21 cynomolgus monkeys were tested by flow cytometry for binding of IgM and IgG to peripheral blood mononuclear cells (PBMC) from wild-type (WT) and GT-KO pigs. The sera were also tested for complement-dependent cytotoxicity to WT and GT-KO PBMC. Anti-WT IgM and IgG were found in 100% and 95%, respectively, and anti-GT-KO IgM and IgG in 76% and 66%, respectively, in the sera of the monkeys tested ($P < 0.01$). Whereas 100% of sera were cytotoxic to WT PBMC, only 76% were cytotoxic to GT-KO PBMC, and the level of cytotoxicity was significantly less ($P < 0.01$). Although the incidence and cytotoxicity of antibodies in monkey sera to GT-KO pig PBMC are significantly less than to WT PBMC, approximately three-quarters of the monkeys tested had cytotoxic antibodies to GT-KO PBMC. This incidence of cytotoxicity is significantly higher than that found in baboons and humans, suggesting the baboon may be an easier and possibly more suitable model to study antibody-mediated rejection of transplanted GT-KO pig organs and cells.

Introduction

The recent availability of pigs homozygous for α1,3-galactosyltransferase gene-knockout (GT-KO) [1,2] has enabled pig-to-nonhuman primate organ and cell transplantation to be carried out in the absence of expression of the pig Galα1,3Gal (Gal) epitopes that are known to be important targets for primate antipig antibodies [3–6]. Hyperacute and acute humoral xenograft rejection (AHXR) are largely caused by these anti-Gal antibodies. An AHXR is believed to be a consequence of antibody binding to vascular endothelial cells, whereby anti-Gal as well as anti-nonGal antibodies (i.e. antibodies against targets other than Gal antigens) in the recipient are of importance. A varying incidence of anti-nonGal antibodies has been reported humans by Zhu [6–8]. These anti-nonGal antibodies appear to be associated with rejection or injury of pig organs or cells in nonhuman primates [9–10, and Ezzelarab M, *et al.*, submitted for publication].

In experimental settings, macaque monkeys (i.e. cynomolgus and rhesus monkeys) and baboons are frequently used as recipients to study the survival of pig organs and cells. Possible differences in anti-nonGal antibodies between species are of importance as they may have implications for suitability of the preclinical model.

There have hitherto been no definitive reports on the incidence of anti-nonGal antibodies in cynomolgus monkeys, nor of the extent of cytotoxicity associated with these antibodies toward pig cells. We here report on the incidence and cytotoxicity of preformed antibodies directed to nonGal antigens on GT-KO pig peripheral blood mononuclear cells (PBMC) in immunologically naïve (not intentionally sensitized) cynomolgus monkeys. We also compare the incidence of anti-nonGal antibodies and their cytotoxicity in cynomolgus monkeys with results obtained by our group in baboons and humans.

Methods

Animals

Serum samples were prepared from blood collected from 21 immunologically naïve cynomolgus monkeys (*Macaca fascicularis*) of all blood groups (A, B, AB, O) purchased from different suppliers. To detect the presence of antipig and anti-nonGal antibodies in these samples, PBMC were collected from GT-KO and unmodified (wild-type, WT) pigs, and used as targets in flow cytometry and cytotoxicity assays. The pigs were obtained from Revivicor, Inc. (Blacksburg,VA, USA) and were of Large White/Landrace/Duroc cross-breed [1]; the GT-KO pigs differed from the WT pigs only with regard to the absence of Gal epitopes, which was confirmed by several different methods [1].

All animal care procedures were in accordance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86–23, revised 1985).

Isolation of Porcine PBMC

Peripheral blood mononuclear cells from several different pigs (WT and GT-KO) were isolated from heparinized blood; the PBMC were not pooled, but PBMCs from a single pig were used for testing of several sera. Briefly, heparinized blood samples were subjected to gravity centrifugation at 560 *g* for 5 min at 20 °C. Plasma from each sample was then removed, and the remaining cells were resuspended in phosphate-buffered saline (PBS; Sigma, St Louis, MO, USA). The final dilution of blood to PBS was 2:1. These cells were then layered slowly and carefully over 5 ml Ficoll-Paque PLUS (Amersham Biosciences, Piscataway, NJ, USA) in 15 ml Falcon conical tubes (Becton Dickinson, Franklin Lake, NJ, USA). The cells were gravity centrifuged at 440 *g* for 30 min at 20 °C, and the buffy coat containing the PBMC was isolated and collected in a separate tube. The total mononuclear cell fraction was then washed twice, filtered through a mesh and resuspended with Roswell Park Memorial Institute (RPMI) 1640 media (Gibco, Grand Island, NY, USA), and live cells were counted using trypan blue (Gibco) under the light microscopy.

Flow cytometry

Isolated PBMC were resuspended in FACS-buffer [PBS containing 1% bovine serum albumin (Gibco) and 0.1% NaN_3] to a final cell concentration of 12.5×10^6 cells/ml. One million PBMC were then removed and incubated with 20 μl of heat-inactivated monkey serum (at 56 °C for 30 min), diluted in FACS buffer to 20% serum final concentration, for 30 min at 4 °C. After incubation, the cells were washed twice in 2 ml FACS buffer, vortexed, and centrifuged at 560 *g* for 5 min. Cells were then resuspended in 10 μl of 10% goat serum in FACS buffer to prevent nonspecific binding, and incubated further with 10 μl fluorescein isothiocyanate (FITC)-conjugated goat antihuman IgG (γ chain-specific) at 1:50 dilution, or with IgM (μ chain-specific) at 1:200 dilution, (Zymed Laboratories, San Francisco, CA, USA) for 30 min in the dark at 4 °C. Antibodies bound to live cells were analyzed using FACScan (Becton Dickinson, Mountain View, CA, USA). Dead cells were excluded from the analysis by forward-scatter and propidium iodide staining (10 μl added to each sample prior to FACS analysis). Binding of a serum sample was considered positive when the mean fluorescence intensity (MFI) was higher than the MFI of the control (cells and secondary antibody alone) plus two standard deviations of the mean control level.

Complement-dependent cytotoxicity assay

Heat-inactivated monkey serum samples ($n = 21$) were diluted fourfold in RPMI 1640 media (+10% FBS; Gibco) to final concentrations of 50%, 12.5%, 3.125%, and 0.78%. As an interplate control, serum from a single human was diluted in the same manner and its complement-dependent cytotoxicity was tested on all plates. Cells were loaded into 96-well flat bottom plates at a cell density of 1×10^6 cells/well in 10% FBS in RPMI 1640 media. The plates were centrifuged at 1800 rpm for 7 min, the supernatant discarded, and the cells were incubated with 100 μl of diluted monkey or human serum (control) for 30 min at 4 °C. Control wells, no serum added, were loaded with 100 μl of medium only. After incubation, the cells were washed (200 μl RPMI 1640 +10% FBS), centrifuged at 1800 rpm for 7 min, the supernatant discarded, and further incubated with 100 μl of 10% rabbit HLA-ABC serum (Sigma), as a source of complement, for 45 min at 37 °C. Negative control wells

(100% live cells) were those to which only complement was added; positive control wells (0% live cells) were those in which a detergent (polyoxyethylene sorbitan monolaurate; Sigma) was added to lyze all cells. Additional negative controls were employed; these comprised either of cells alone or cells and serum.

Following the incubation, the cells were washed and resuspended in 100 μl of RPMI 1640 (+10% FBS) and 20 μl of MTS (CellTiter 96®, Aqueous One Solution Reagent; Promega, Madison, WI, USA) and incubated for 15 h, for the development of color, at 37 °C in a 5% CO_2-humidified atmosphere. Cell viability was assessed by the capacity of live cells to reduce tetrazolium salts into formazan, and the color produced was measured (absorbance) using a plate reader (Bio-Tek Instruments, Winooski, VT, USA) at a wavelength of 450 nm. The assumption made in this assay was that a cell that is incapable of producing color is a dead cell. Cell viability was calculated using the following formula:

$$\%\ \text{live cells} = [(A - B)/(C - B)] \times 100,$$

where A equals the absorbance of cells (+ serum and complement), B equals the absorbance of cells (+ detergent), and C equals the absorbance of cells (+ complement).

Results were expressed as the percentage of dead cells (% cytotoxicity = 100 − % viability). Complement-dependent cytotoxicity values of the varying serum concentrations (50%, 12.5%, 3.125%, and 0.78%) were calculated, and a curve was generated for each monkey sample. Lysis of PBMC of <10% was considered of doubtful relevance.

Data analysis

Analysis of indirect immunofluorescence intensity was accomplished by CELL QUEST software and converted into MFI by WINMDI software. Statistical analysis of data was performed using Mann–Whitney *U*-tests. Correlation of cytotoxicity and MFI was assessed by linear regression analysis. Significance at the 95% or the 99% level was calculated using PRISM-4 software (Graphpad Software; San Diego, CA, USA).

Results

Reactivity of Cynomolgus Monkey Serum Samples to PBMC from WT and GT-KO Pigs

Binding of monkey IgM and IgG to pig PBMC by flow cytometry

The presence of preformed xenoreactive antibodies in sera from immunologically naïve (not intentionally sensitized) cynomologus monkeys was assessed by flow cytometry. All 21 serum samples had a detectable IgM binding to WT PBMC, and 16 of 21 sera (76%) showed some binding to PBMC from GT-KO pigs (Fig. 1a). A similar finding was observed with IgG binding; 20 of 21 samples (95%) demonstrated IgG binding to WT PBMC, while only 14 (67%) bound GT-KO cells (Fig. 1a). In other words, with the flow cytometry assay employed in these studies, the incidence of binding of monkey IgM and IgG antibodies to GT-KO PBMC was 76% and 70%, respectively, of that to WT PBMC.

The mean MFI of IgM binding to WT PBMC and to GT-KO PBMC was determined to be 12.14 ± 3.54 and 6.66 ± 2.45, respectively; this mean MFI level of IgM binding to WT PBMC was significantly higher than that to GT-KO PBMC ($P < 0.01$, Mann–Whitney *U*-test) (Fig. 1b). The mean MFI of IgG binding to WT PBMC was 9.96 ± 6.34, and the mean binding of IgG to GT-KO PBMC was 4.30 ± 1.17, the difference also achieving statistical significance ($P < 0.01$) (Fig. 1b).

These results would suggest that (i) the greater binding to WT PBMC was because of Gal-specific antibodies, and (ii) the residual binding to GT-KO PBMC was because of the presence of antibodies to nonGal-specific antigens.

Complement-dependent cytotoxicity of monkey sera to pig PBMC

After establishing that monkey serum can contain anti-nonGal antibodies, it was important to determine whether these antibodies can cause lysis of cells from GT-KO pigs. Lysis was assessed by using a classical two-step complement-dependent cytotoxicity assay. The cytotoxicity of the monkey serum samples was tested at various concentrations (50%, 12.5%, 3.125%, and 0.78%). In three cases, there was insufficient serum to include a 50% concentration in the assay.

The result of the assay was considered acceptable when the slope of the human control curve was within the 95% confidence limit of the mean of >25 determinations. Cytotoxicity results from 19 out of 21 sera fell within this confidence interval; however, there were no statistically significant differences whether 19 or 21 sera were included in the data analysis. (The sensitivity of the assay was such that only lysis of >10% was considered positive.)

The strength of serum cytotoxicity to both WT and GT-KO PBMC varied among the different monkeys. At 12.5% dilution, all the sera were cytotoxic to WT PBMC, whereas only 76% of these samples were lytic to PBMC from GT-KO pigs (Fig. 2a). Furthermore, these sera caused greater lysis of PBMC from WT pigs than from GT-KO pigs ($P < 0.01$) (Fig. 2a and b); at 12.5% dilution, the lysis of PBMC was reduced from approximately 54% (WT) to 30% (GT-KO) (Fig. 2b).

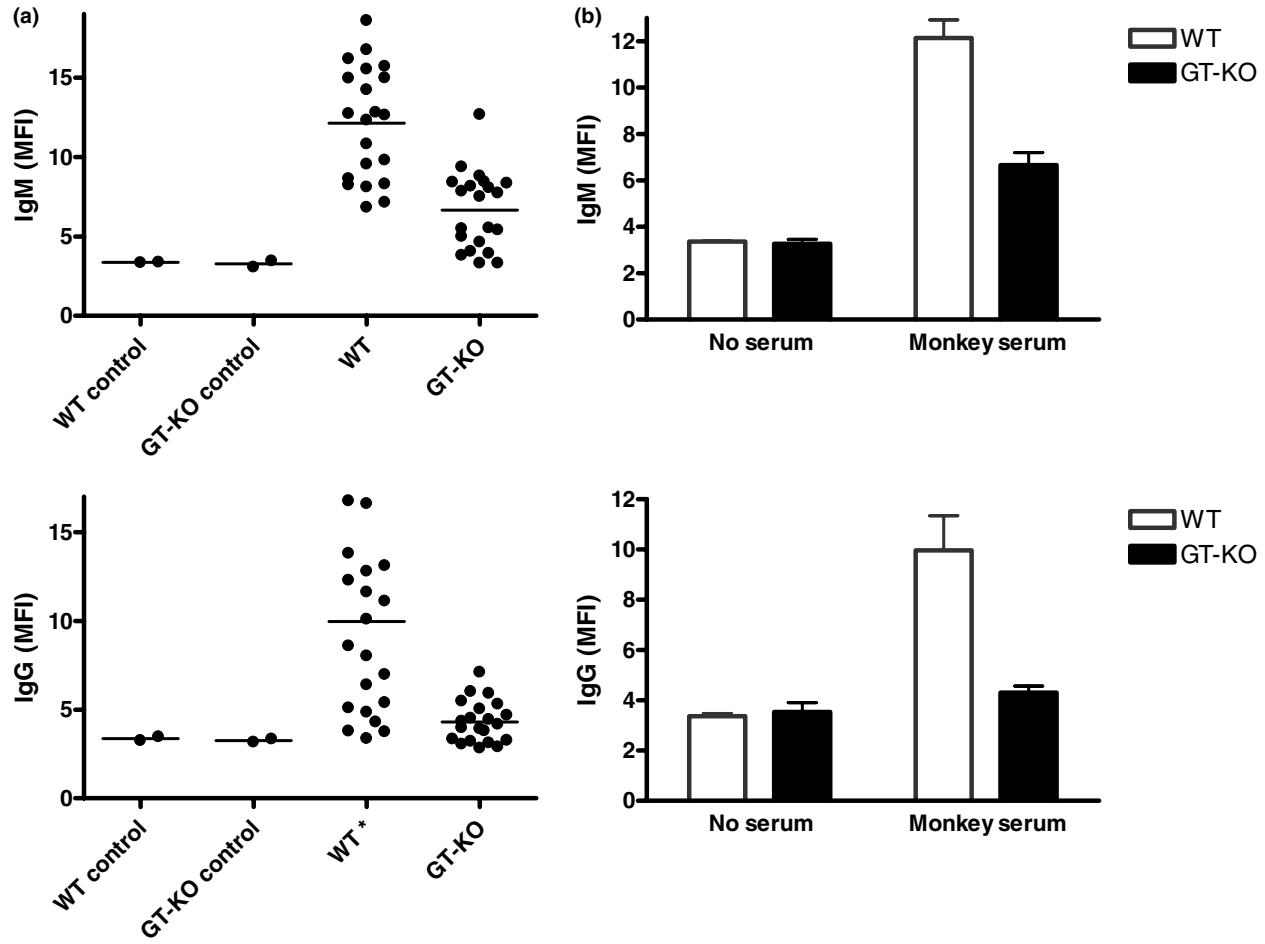

Figure 1 Binding of cynomolgus monkey serum antibodies to wild-type (WT) and α1,3-galactosyltransferase gene-knockout (GT-KO) pig cells (MFI = mean fluorescence intensity). (a) Distribution of serum reactivity of 21 monkeys against WT or GT-KO peripheral blood mononuclear cells (PBMC), IgM (top) and IgG (bottom). Mean reactivity of each group is indicated by a line. Control is fluorescein isothiocyanate (FITC)-conjugated antibody and pig cells only. (b) Mean reactivity (MFI levels ± SEM) of serum samples ($n = 21$) against WT or GT-KO PBMC, IgM (top) and IgG (bottom). Statistically higher reactivity of both IgM and IgG against WT PBMC versus GT-KO PBMC was observed ($P < 0.01$).

We concluded that approximately three quarters of naïve monkey sera tested in this study contained anti-nonGal antibodies that are cytotoxic to PBMC from GT-KO pigs, though the extent of cell lysis is generally less than to WT pig PBMC.

Correlation between binding of IgM or IgG and serum cytotoxicity

To investigate if one of the immunoglobulin isotypes was more associated with cytotoxicity, correlation between cytotoxicity and binding (MFI) was assessed. Correlations were found between IgM binding to both WT and GT-KO PBMC and cytotoxicity (at 12.5% serum concentration) ($P < 0.02$) (Fig. 3). However, no statistically significant correlation between IgG binding and cytotoxicity to WT or GT-KO PBMC was observed (Fig. 3).

Discussion

The incidence of binding of monkey serum IgM and IgG isotypes to PBMC from GT-KO pigs was significantly less when compared with binding to WT PBMC. However, we demonstrated that 76% of the tested monkey sera caused lysis of GT-KO PBMC (compared with 100% lysis of WT PBMC), although the extent of cell lysis associated with anti-nonGal antibodies was significantly less. Even though this confirms the potential advantage of using GT-KO donor pigs for transplantation into cynomolgus monkeys, nevertheless, approximately three quarters of the monkeys had IgM with or without IgG binding to GT-KO PBMC; as these anti-nonGal antibodies are cytotoxic, they are likely to be associated with the development of AHXR.

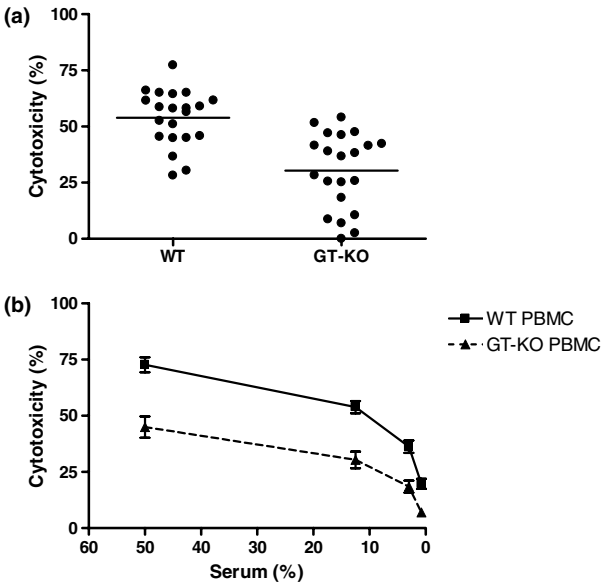

Figure 2 (a) Ability of individual monkey serum samples at 12.5% dilution to cause lysis of PBMC from WT or GT-KO pigs; mean complement-dependent cytotoxicity is indicated by lines. Significantly, higher lysis of unmodified (WT) PBMC was observed ($P < 0.01$). (b) Mean cytotoxicity of monkey serum samples (at various dilutions) against PBMC from either WT or GT-KO pigs.

One possible limitation of our study is that only PBMCs were used as target cells, whereas vascular endothelial cells might have provided different, or more biologically relevant, nonGal epitopes, although there is no definite evidence for this. We are currently investigating whether there are differences between antipig antibody binding and cytotoxicity to PBMC and vascular endothelial cells; preliminary results suggest that there is little difference and, indeed, binding to PBMC may be higher (H. Hara et al., unpublished data, Starzl Transplantation Institute, Pittsburgh, USA). Furthermore, although PBMC were drawn from WT pigs from the same herd and from GT-KO pigs from three closely-related clones, neither WT nor GT-KO PBMC were pooled; the possibility of variable Gal and nonGal epitope expression between the PBMC cannot be ruled out, although this is likely to have been minor.

In other studies by our group, 43% of human (H. Hara, preliminary data, Starzl Transplantation Institute, Pittsburgh, USA) and 32% of baboon [Ezzelarab M, *et al.*, submitted for publication] sera were demonstrated to have an IgM binding to GT-KO PBMC, which is significantly less than the 76% IgM binding of cynomolgus monkey sera that we report here ($P < 0.01$). Both human and monkey sera showed an incidence of 67% IgG binding to GT-KO PBMC, compared with an incidence of only 9% of baboon IgG ($P < 0.01$). The levels of IgG and IgM binding of all three species are shown in Fig. 4. Furthermore, whereas approximately 50% of both human and baboon sera demonstrated cytotoxic antibodies against GT-KO PBMC, the incidence is 76% in monkey sera ($P < 0.01$) (Fig. 5). These results suggest that monkeys may have stronger antibody-mediated cytotoxic responses to GT-KO pig grafts than humans and baboons.

In experimental settings, GT-KO organs have been transplanted into immunsuppressed baboons; heterotopic heart graft failure occurred after 2–6 months [11] and recipients with life-supporting pig kidneys died from complications of the regimen within 3 months, although in some transplant kidney function continued [12]. Whereas anti-Gal antibodies could not have been part of this rejection process, anti-nonGal antibodies could possibly have been involved. These findings are supported by a study from Brandl *et al.* in an hDAF transgenic pig-to-baboon heart transplantation model using the soluble Gal saccharides to prevent anti-Gal antibody-mediated hyperacute rejection; this center reported that nonGal antibody titers were increased, and this increase was associated with the development of AHXR [9].

To our knowledge, there are no reports of transplantation of GT-KO organs or cells in cynomolgus monkeys. However, there is an evidence that anti-nonGal antibodies are associated with AHXR in cynomolgus monkeys; Lam *et al.* [10] demonstrated that, when hDAF-transgenic pig hearts were transplanted into cynomolgus monkeys under the soluble Gal saccharide-based therapy (to adsorb or 'neutralize' anti-Gal antibodies), anti-nonGal antibody levels increased in animals that rejected their grafts.

Although the identity of pig nonGal antigens still remains uncertain [13], there is evidence that anti-*N*-glycolylneuraminic acid antibodies, also known as Hanganutziu-Deicher (HD) antibodies, are present in human serum [8,14]. As pigs express *N*-glycolylneuraminic acid on their cells, this may, in part, account for human anti-nonGal antibody binding to GT-KO PBMC. However, as baboons and monkeys express HD antigens themselves (like the pig), anti-HD antibodies do not contribute to the anti-nonGal antibodies found in these nonhuman primates species. The higher level of anti-nonGal IgM in monkeys detected in our studies compared with that in humans and baboons cannot be fully explained, but it is probably related to differences in the microbial flora that populate the gastro-intestinal tract or to other infectious agents to which these various species have been exposed. Also, the age and geographic origin, and whether wild-caught or captive-bred, of the monkeys from which sera were drawn may have played a role in this [15–17], but unfortunately this information was not available to us for sufficient monkeys

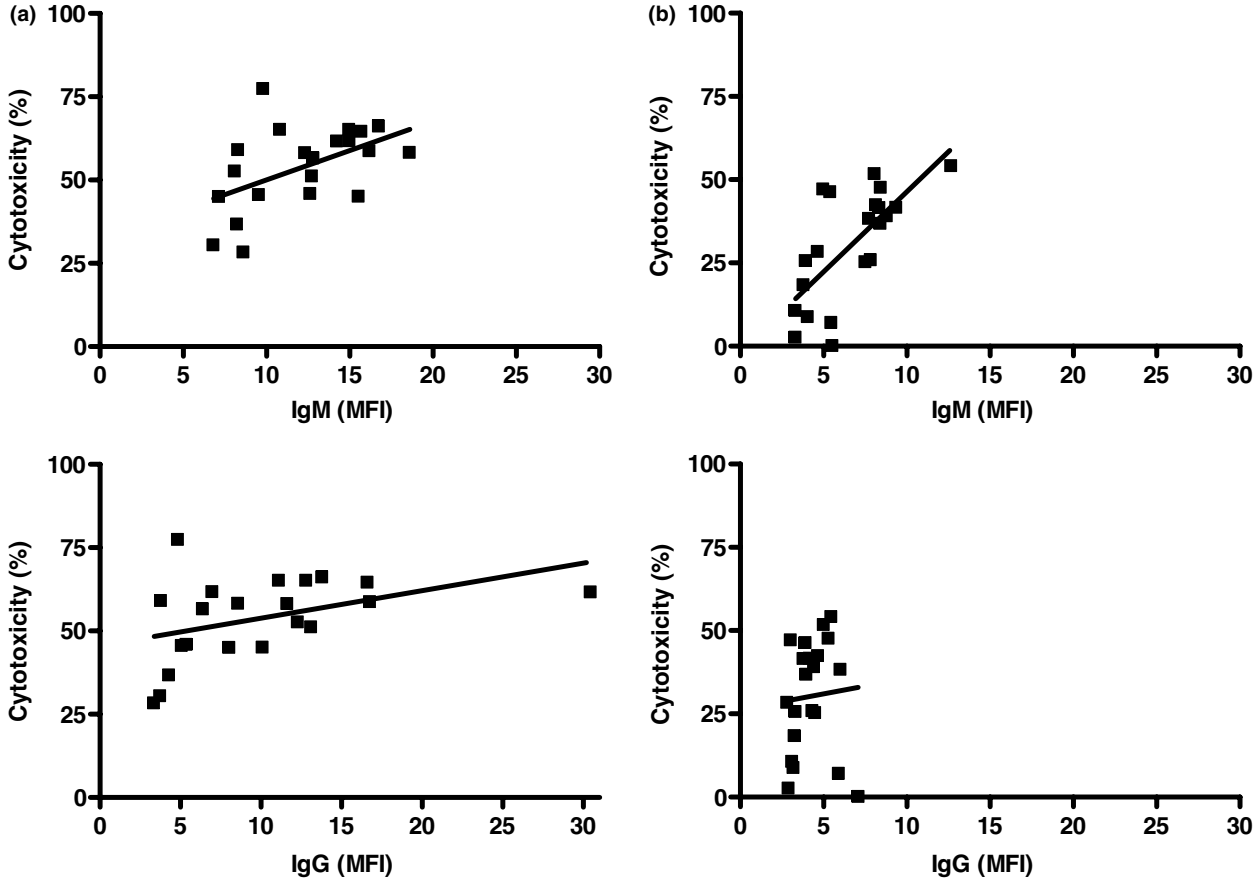

Figure 3 Linear regression of monkey serum cytotoxicity (at 12.5% serum concentration) and immunoglobulin isotype binding to (a) WT and (b) GT-KO PBMC ($n = 21$). Correlations were found between IgM binding (top) to both WT and GT-KO PBMC and cytotoxicity; no statistically significant correlation was observed between IgG binding (bottom) to WT and GT-KO PBMC and cytotoxicity.

to draw conclusions in this respect. The target(s) for these anti-nonGal antibodies needs to be ascertained.

The observed differences in the levels of anti-nonGal IgM and IgG and cytotoxicity between the species may be, at least in part, related to the relatively small numbers in each group. A study involving sera from larger numbers of each species may find less variation. A factor that must also be considered is the isotypes of anti-nonGal IgG in the three species, which we did not explore. Although not identified in this study, the ability of the IgG isotype to activate complement may have been an important factor in the correlation between antibody and cytotoxicity. However, if monkeys are confirmed to have higher levels of anti-nonGal IgM, this may be associated with a higher incidence of hyperacute rejection of a transplanted pig organ than in either baboons or humans.

In addition to this potential role in the rejection of GT-KO pig organs, anti-nonGal antibodies may also be important following the adult pig islet transplantation in primates. As adult pig islets are known to express little or no Gal [18–21], anti-nonGal antibodies are likely to be involved in any antibody-mediated rejection process [22–24].

It has sometimes been considered that the baboon is a more difficult preclinical model of xenotransplantation than the macaque monkey. Our data suggest that antibody-mediated xenograft rejection of GT-KO pig organs or cells may be more vigorous in cynomolgus monkeys than in baboons or humans. With regard to the cytotoxicity associated with anti-nonGal antibody binding, the baboon may be closer to the human than the cynomolgus monkey, and therefore may be the preferred experimental recipient of GT-KO pig grafts.

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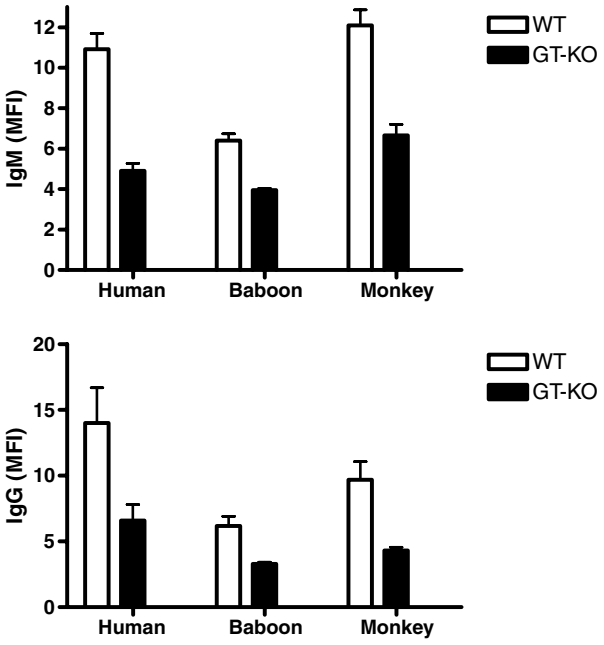

Figure 4 Comparison of binding of human, baboon, and cynomolgus monkey preformed xenoreactive antibodies to WT and GT-KO pig PBMC. Mean reactivities of human ($n = 21$), baboon ($n = 56$), and monkey ($n = 21$) sera against WT or GT-KO PBMC are shown, IgM (top) and IgG (bottom).

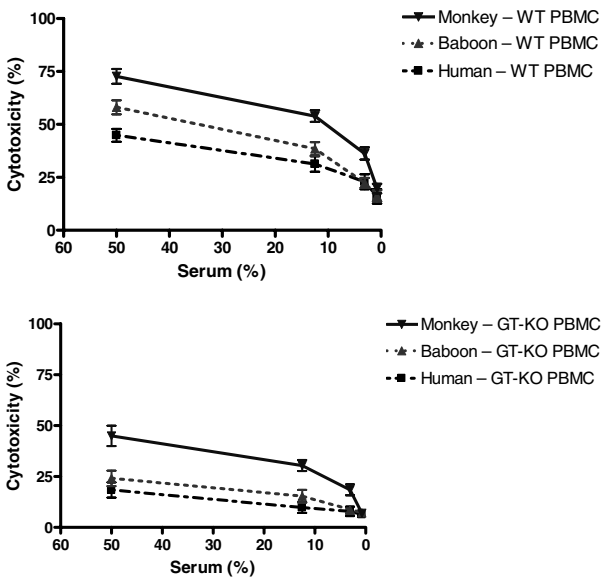

Figure 5 Comparison of mean cytotoxicity of human ($n = 21$), baboon ($n = 39$), and monkey ($n = 19$) serum samples against PBMC from either WT (top) or GT-KO (bottom) pigs.

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