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ORIGINAL ARTICLE

In vitro investigation of pig cells for resistance to human antibody-mediated rejection

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Summary

Although human complement-dependent cytotoxicity (CDC) of α1,3-galactosyltransferase gene-knockout (GTKO) pig cells is significantly weaker than that of wild-type (WT) cells, successful xenotransplantation will require pigs with multiple genetic modifications. Sera from healthy humans were tested by (i) flow cytometry for binding of IgM/IgG, and (ii) CDC assay against peripheral blood mononuclear cells and porcine aortic endothelial cells from five types of pig – WT, GTKO, GTKO transgenic for H-transferase (GTKO/HT), WT transgenic for human complement regulatory protein CD46 (CD46) and GTKO/CD46. There was significantly higher mean IgM/IgG binding to WT and CD46 cells than to GTKO, GTKO/HT, and GTKO/CD46, but no difference between GTKO, GTKO/HT, and GTKO/CD46 cells. There was significantly higher mean CDC to WT than to GTKO, GTKO/HT, CD46, and GTKO/CD46 cells, but no difference between GTKO and GTKO/HT. Lysis of GTKO/CD46 cells was significantly lower than that of GTKO or CD46 cells. CD46 expression provided partial protection against serum from a baboon sensitized to a GTKO pig heart. GTKO/CD46 cells were significantly resistant to lysis by human serum and sensitized baboon serum. In conclusion, the greatest protection from CDC was obtained by the combination of an absence of Gal expression and the presence of CD46 expression, but the expression of HT appeared to offer no advantage over GTKO. Organs from GTKO/CD46 pigs are likely to be significantly less susceptible to CDC.

Introduction

The availability of pigs homozygous for α1,3-galactosyltransferase gene-knockout (GTKO) [1,2] has enabled pig-to-baboon organ transplantation to be carried out in the absence of Galα1,3Gal (Gal) epitopes that are known to be important targets for primate anti-pig antibodies [3–6]. Transplantation of hearts [7] and kidneys [8] from GTKO pig into immunosuppressed baboons was followed by relatively prolonged graft survival. Heart graft survival is currently limited by the development of a thrombotic microangiopathy that may be a form of delayed antibody-mediated rejection.

However, it is clear that anti-non-Gal antibodies can be associated with the rejection or injury of GTKO organs in baboons [9]. The incidence and complement-dependent cytotoxicity (CDC) of preformed antibodies to GTKO pig peripheral blood mononuclear cells (PBMC) are significantly less than those to wild-type (WT) in humans [10], baboons [11], and monkeys [12]. However, approximately 50% of primates had cytotoxic antibodies to GTKO PBMC. These may be associated with early rejection of GTKO organs [9,13]. Further genetic modification of the organ-source pig would be advantageous to reduce antibody binding and/or CDC.

The addition of the H-transferase gene to GTKO cells (GTKO/HT), increasing expression of the H(O) antigen (the universal human donor antigen), may increase protection by reducing antibody-binding and CDC [14–17]. Alternatively, the expression of a complement-regulatory protein, such as CD46, is known to protect pig cells from CDC [18–21].

During transplantation, an organ is subjected to various insults, such as ischemia and reperfusion, that result in the activation of the endothelium [22]. Studying activated pig aortic endothelial cell (PAEC) provides additional information that is perhaps more indicative of the *in vivo* situation, especially in the acute phase.

We investigated antibody binding and CDC of human sera to WT, GTKO, GTKO/HT, CD46, and GTKO/CD46 PBMC and PAEC. Furthermore, we investigated the effect of activation of the PAEC on IgM/IgG binding and CDC.

Methods

Human serum and PBMC donors

Serum was collected from 16 healthy human volunteers of all ABO blood types who had no history suggesting previous exposure to pig antigens or to alloantigens (i.e., no previous pregnancies, blood transfusions, or organ allotransplants). Pooled healthy human sera (including all ABO blood types) were also used. The sera were stored at −80 °C. Decomplementation was carried out by heat-inactivation for 30 min at 56 °C. PBMC were obtained from three healthy unrelated human volunteers who were of blood type O. Participants gave informed consent as per the guidelines of the Institutional Review Board of the University of Pittsburgh.

Sensitized baboon serum

Serum from one sensitized baboon was also used for IgM/IgG binding and CDC. This baboon had previously received a heart transplant from a GTKO pig without immunosuppression. The heart was electively excised after 150 min and remnants of the pig aorta and pulmonary artery were left *in situ* for 10 weeks [11].

Pig cell sources

Peripheral blood mononuclear cells or PAEC were collected from WT, GTKO, GTKO/HT, CD46, and GTKO/CD46 pigs (all provided by Revivicor, Inc., Blacksburg, VA, USA). Two to three pigs from each type were used for these experiments. They were all of blood type non-A(O). The WT and GTKO pigs were of Large White/Landrace/Duroc cross-breed, but were not from identical clones. The GTKO/HT, CD46, and GTKO/CD46 pigs were derived from cross-breeding between different herds of Large White pigs.

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 PBMC

Peripheral blood mononuclear cell from pigs or humans were isolated, as previously described [10]. PBMC were resuspended in FACS buffer (PBS containing 1% BSA and 0.1% NaN_3) for flow cytometry or in cytotoxicity medium (RPMI culture medium; Invitrogen, Carlsbad, CA, USA) containing 10% controlled process serum replacement-type 3 (CPSR-3, Sigma, St. Louis, MO, USA), 1% HEPES buffer (Invitrogen), and 100 IU/ml penicillin–100 μg/ml streptomycin (Invitrogen) for CDC assay.

Vascular endothelial cells

Pig aortic endothelial cell were obtained from freshly harvested porcine aortas by treatment with 0.05% collagenase B (Roche Applied Science, Indianapolis, IN, USA). The cells were collected and washed with washing medium [RPMI containing 10% heat-inactivated bovine serum (Invitrogen) to inactivate the collagenase], and then were cultured in PAEC culture medium (medium 199, Invitrogen) containing 10% heat-inactivated FBS (Sigma) and antibiotic–antimycotic (Invitrogen) and endothelial growth factor (30 μg/ml, BD Biosciences, San Jose, CA, USA).

Human aortic endothelial cells (HAEC), purchased from Cambrex (Walkersville, MD, USA), were cultured with endothelial growth medium-2 (Cambrex). Both PAEC and HAEC were grown to confluence into a collagen I-coated 25 cm^2 or 75 cm^2 tissue culture flask (BD) and used for experiments between passages 2–6. Activation of subconfluent PAEC was carried out by culture in recombinant porcine IFN-γ (400 unit/ml; Serotec, Raleigh, NC, USA) for 48 h.

Cell staining for identification of antigens (Gal, H) and CD46, CD31, and SLA Class II

The PBMC, PAEC, and HAEC were diluted to 10^5 cells per tube in FACS buffer. Surface expression of the Gal and H antigens and CD46 was measured by direct immunofluorescence using FITC-conjugated isolectin B4 from *Bandeiraea simplicifolia* (BS-IB4, Sigma), FITC-conjugated lectin from *Ulex europaeus* (UEA-I, Sigma), FITC-conjugated mouse anti-human CD46 mAb (clone MEM-258; Serotec),

and FITC-conjugated mouse IgG1 isotype control (clone W3/25; Serotec). Cells were incubated for 30 min at 4 °C. Isotype-matched mAb or staining buffer alone (for BS-IB4, UEA-1) were used as negative controls. To confirm activation of endothelial cells, PAEC were stained with purified anti-pig swine leukocyte antigen-DR (SLA-DR) mAb (clone 1053h2-18-1; BD) or purified mouse IgG2aκ isotype control (clone G155-178; BD), followed by staining with FITC-conjugated anti-mouse IgG2a/2b mAbs (clone R2-40; BD). Purity of PAEC was confirmed to be >90% by staining with PE-conjugated mouse anti-rat CD31 mAbs (clone;TLD-3A12; BD) or PE-conjugated mouse IgG1κ isotype control (clone X40; BD).

Binding of IgM and IgG to pig PBMC or PAEC

Binding of xenoreactive antibody to pig cells was measured as previously described [10]. Briefly, 20% or serial diluted heat-inactivated human serum or FACS buffer (control) was incubated with 10^5 target cells for 30 min at 4 °C. To prevent nonspecific binding, 10% goat serum was added after washing twice. Detection of IgM or IgG binding was performed by further incubating with FITC-conjugated goat anti-human IgM (μ chain-specific) and IgG (γ chain-specific) (Invitrogen) for 30 min at 4 °C. Data acquisition was performed with BD™ LSR II flow cytometer (BD). Binding of IgM and IgG was assessed using relative mean fluorescence intensity (MFI) which was obtained as follows:

$$\text{Relative MFI} = (\text{actual MFI value})/(\text{MFI obtained using secondary antibody only, in the absence of serum})$$

Complement-dependent cytotoxicity assay using chromium[51]

The CDC assay was carried out as previously described [23]. Briefly, target cells were prepared from PBMC or PAEC, and incubated with ^{51}Cr (50 μCi for every 1×10^6 PBMC or 100 μCi for every 1×10^6 PAEC) for 60 min at 37 °C. Labeled cells were washed twice. On a 96-well round-bottom plate (Corning, Corning, NY, USA), 10^4 ^{51}Cr-labeled cells suspended in cytotoxicity medium were loaded into each well, and incubated with heat-inactivated human or sensitized baboon serum at various dilutions for 30 min at 37 °C. After incubation, the cells were further incubated with 5% rabbit HLA-ABC serum (Sigma), as a source of complement, for 45 min at 37 °C.

Cell killing was calculated as follows: % cytotoxicity = $([A - C]/[B - C]) \times 100$, where A represents the experimental release (cpm in the supernatant from target cells incubated with serum and complement), B is the maximal release (cpm released from target cells lysed with 4%Titron), and C is the minimal release (cpm in the supernatant from target cells incubated with complement or medium only). CDC values at the varying serum concentrations were calculated, and a curve was generated for each sample.

Statistical methods

Values are presented as mean ± SEM. The statistical significance of differences was determined by Student's t-test or nonparametric tests, as appropriate. The statistical tests were carried out using GraphPad Prism version 4 (Graphpad Software, San Diego, CA, USA). Differences were considered to be significant at $P < 0.05$.

Results

Expression of H antigen on cells from GTKO/HT pig

Although WT pigs expressed Gal on PBMC and PAEC, GTKO and GTKO/HT pigs and humans did not express Gal on PBMC or on aortic endothelial cells (Fig. 1A[a] and B[a]). GTKO/HT pig PBMC expressed high levels of H antigen on their surface compared with humans and GTKO and WT pigs (Fig. 1A[b]), and GTKO/HT PAEC expressed higher levels of H antigen than WT, GTKO PAEC, and HAEC (Fig. 1B[b]). However, GTKO PBMC and, particularly, PAEC expressed moderately high levels of the H antigen. The expression of the H antigen on GTKO PBMC and PAEC was much higher than on WT pig cells.

Binding of human IgM and IgG to PBMC and PAEC from WT, GTKO, and GTKO/HT pigs

Relative mean MFI of IgM and IgG binding to WT PBMC was significantly higher than that to GTKO and GTKO/HT PBMC (IgM 5.6 vs. 2.3, 2.5; IgG 15.7 vs. 6.0, 8.3) ($P < 0.01$) (Fig. 2A) and PAEC (IgM 4.6 vs. 1.1, 1.3; IgG 6.1 vs. 1.4, 1.7) ($P < 0.01$) (Fig. 2B). However, there was no significant difference of human IgM/IgG binding to GTKO and GTKO/HT pig PBMC and PAEC. These results indicate that (i) the greater binding to WT cells was because of the presence of Gal-specific antibodies, (ii) the residual binding to GTKO and GTKO/HT cells was because of the presence of antibodies to non-Gal antigens, and (iii) the addition of the HT gene did not decrease antibody binding to GTKO pig PBMC or PAEC.

CDC of sera to pig PBMC and PAEC from WT, GTKO, and GTKO/HT pigs

At all serum concentrations (except 0.78% of PAEC), there was significantly lower CDC to both GTKO and

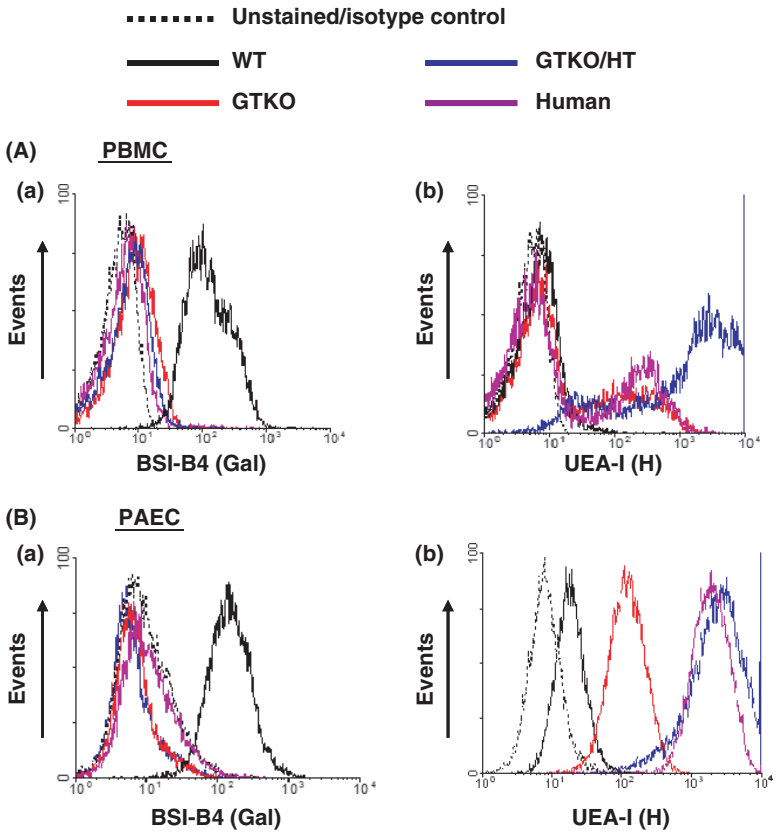

Figure 1 Expression of Gal and H (blood type O) on PBMC (A) and aortic endothelial cells (B) from WT, GTKO, and GTKO/HT pigs and humans. Cells were stained with BSI-B4 (a) or UEA-1 (b). Representative data of three experiments. Dotted lines – unstained or isotype control. Black lines – WT cells. Red lines – GTKO cells. Blue lines – GTKO/HT cells. Purple lines – human cells. Although WT pigs expressed Gal on PBMC and PAEC, GTKO and GTKO/HT pigs and humans did not express Gal on PBMC or on aortic endothelial cells (A[a] and B[a]). GTKO/HT pig PBMC expressed high levels of H antigen on their surface compared with humans and GTKO and WT pigs. GTKO/HT PAEC expressed higher levels of H antigen than WT and GTKO PAEC and HAEC (A[b] and B[b]), but GTKO PAEC expressed a significant level.

GTKO/HT pig PBMC and PAEC than to WT PBMC and PAEC (e.g. at 50% serum concentration, the percent lysis was WT 72% and 47%; GTKO 22% and 9%; GTKO/HT 16% and 11%) ($P < 0.01$) (Fig. 2C,D). However, there was no significant difference in CDC of GTKO and GTKO/HT pig PBMC and PAEC. These results indicate that (i) the greater lysis of WT cells was because of the presence of Gal-specific antibodies, (ii) the residual lysis of GTKO and GTKO/HT cells was because of the presence of antibodies to non-Gal antigens, and (iii) the addition of the HT gene did not decrease CDC of GTKO pig PBMC or PAEC – this is likely to be associated with the considerable expression of the O blood type antigen on GTKO PBMC and PAEC (Fig. 1).

Comparison of IgM/IgG binding and CDC between PBMC and PAEC

Relative mean MFI binding of IgM and IgG to WT, GTKO, and GTKO/HT PBMC was significantly greater than that to the corresponding PAEC ($P < 0.05$) (Fig. 2A,B). CDC of WT, GTKO, and GTKO/HT PBMC was significantly greater than that of the corresponding PAEC ($P < 0.05$) (Fig. 2C,D). These results indicate that (i) binding to PBMC was uniformly greater than to the corresponding PAEC, and (ii) lysis of PBMC was uniformly greater than that of the corresponding PAEC.

Binding to, and CDC of, PAEC are increased after activation of the cells

Activated PAEC (by IFN-γ for 48 h) were used to study IgM/IgG binding and CDC and were compared to nonactivated PAEC from WT, GTKO, and GTKO/HT pigs. SLA class II on all three types of PAEC was up-regulated after activation (Fig. 3A). There was no significant difference in the expression of class II between the three types of PAEC either before or after activation (not shown). We investigated Gal and H antigen expression on activated PAEC. There was no significant increase in either Gal or H expression after activation (Fig. 3B,C).

Binding of IgM and IgG to activated PAEC was significantly increased compared with that to nonactivated PAEC from all three pig types, except binding of IgG to WT PAEC (Fig. 4A,B). CDC was also significantly increased after activation (e.g. at 50% serum dilution, WT 55%; GTKO 30%; GTKO/HT 30%) when compared with quiescent PAEC ($P < 0.01$) (Fig. 4C). There was still

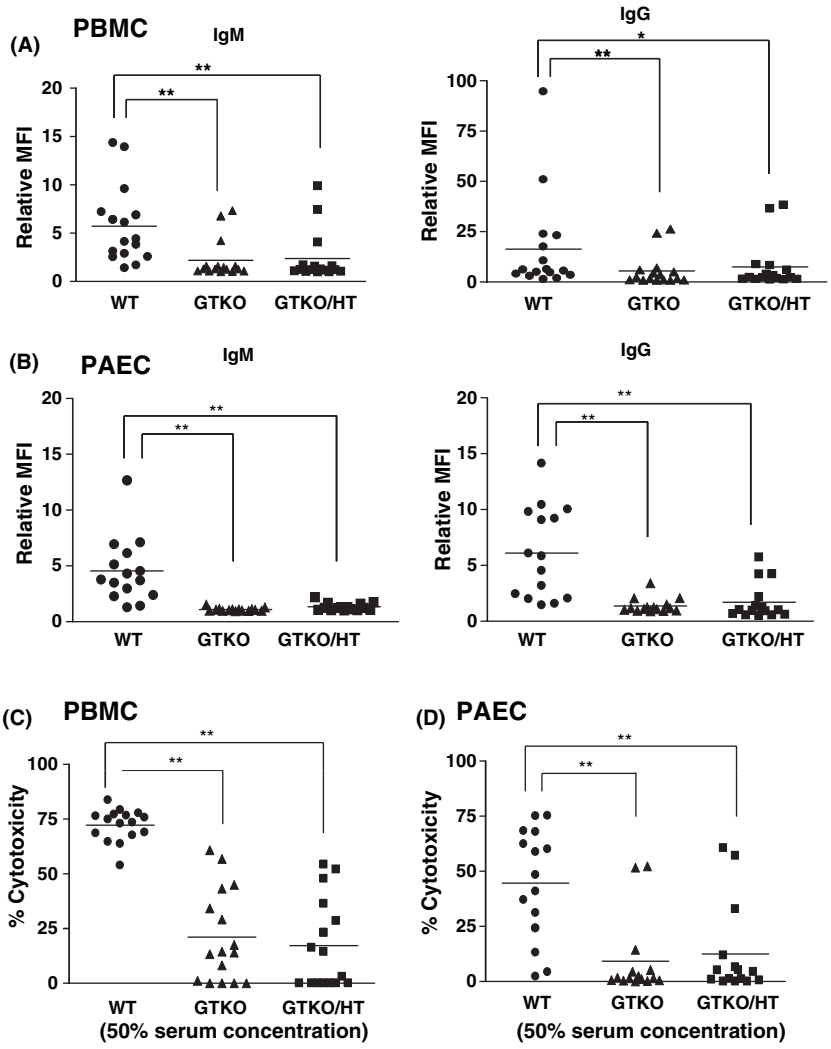

Figure 2 Binding of xenoreactive antibodies and complement-dependent cytotoxicity (CDC) in sera from healthy human volunteers to WT, GTKO, and GTKO/HT PBMC and PAEC. Distribution of 20% serum IgM and IgG reactivity of healthy humans against WT (●), GTKO (▲) and GTKO/HT (■) PBMC (A) (n = 16) and PAEC (B) (n = 15) is shown. Binding of IgM and IgG was assessed using relative MFI with the absence of serum (secondary antibody only) being scored as 1. Mean reactivity of each group is indicated by a horizontal line. IgM and IgG binding to GTKO and GTKO/HT pig PBMC (A) and PAEC (B) were significantly lower than that to WT PBMC. However, there was no significant difference in IgM/IgG binding to GTKO and GTKO/HT pig PBMC and PAEC. Distribution of 50% serum cytotoxicity of healthy humans against WT (●), GTKO (▲) and GTKO/HT (■) PBMC (C) (n = 16) and PAEC (D) (n = 15) is shown. Mean cytotoxicity of each group is indicated by a horizontal line. There was significantly less lysis of both GTKO and GTKO/HT pig PBMC/PAEC than that of WT PBMC/PAEC (C and D). However, there was no significant difference in CDC of GTKO and GTKO/HT pig PBMC/PAEC. (*P < 0.05, **P < 0.01).

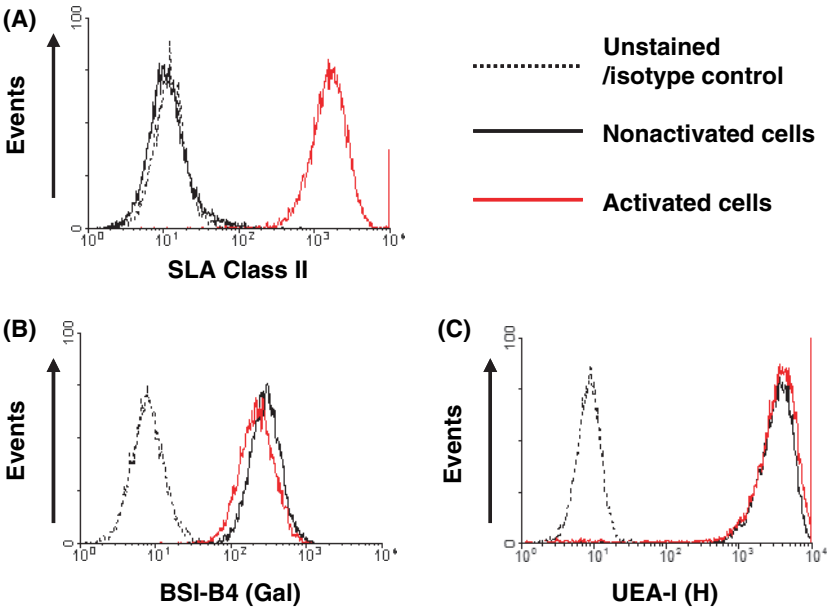

Figure 3 Expression of SLA class II, Gal, and H on PAEC after activation. PAEC were stained for SLA class II from a GTKO pig (A), BSI-B4 from a WT pig (B), or UEA-1 from a GTKO/HT pig (C), before and after activation (by IFN-γ for 48 h). Representative data of three experiments are presented. Dotted lines – unstained or isotype control. Black lines – nonactivated cells. Red lines – activated cells. When PAEC were activated, expression of SLA class II was greatly increased (A), but there was no significant increase in the expression of Gal (B) or H (C) antigen. (N.B. CD46 expression was not increased by activation – not shown.).

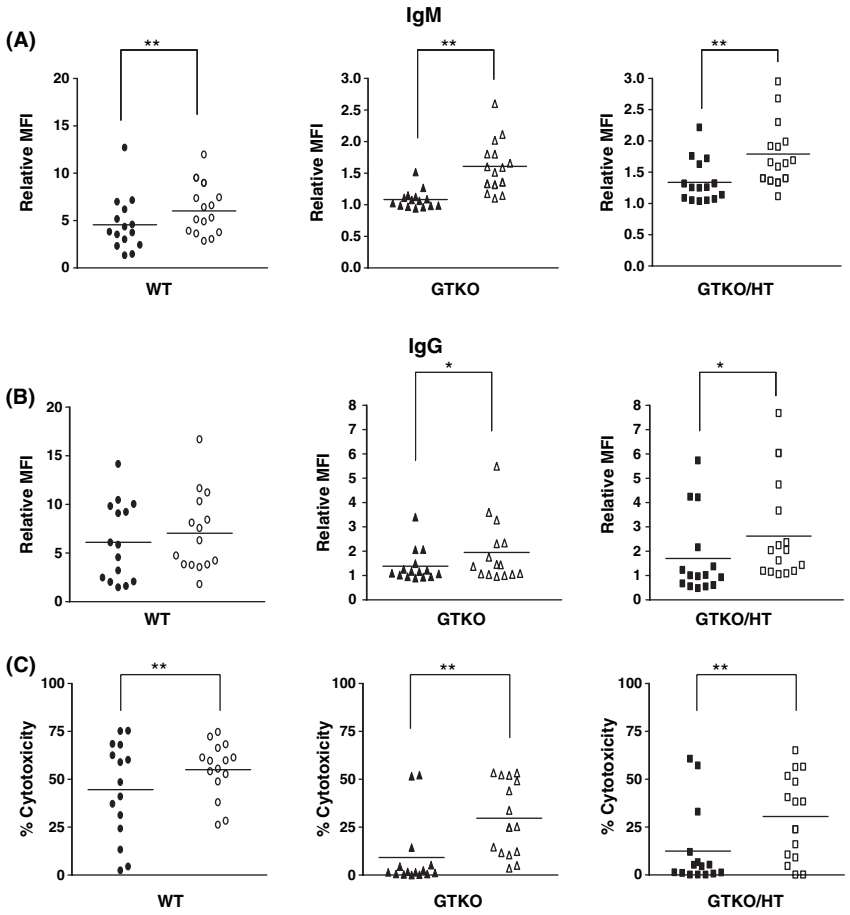

Figure 4 Comparison of IgM/IgG binding and CDC between nonactivated PAEC and activated PAEC from WT, GTKO, and GTKO/HT pigs. IgM (A) and IgG (B) binding and CDC (C) to nonactivated PAEC (●, ▲, ■) or activated PAEC (○, Δ, □) in individual human sera (20% serum concentration for binding assay and 50% serum concentration for CDC assay) ($n = 15$). (Note the differences in scale between WT and GTKO and GTKO/HT data). There was no significant difference in binding and CDC between nonactivated GTKO and GTKO/HT PAEC. When cells were activated (○, Δ, □), there was an increase in antibody binding to all the three cell types compared with nonactivated cells (●, ▲, ■). However, there was still significantly lower binding of IgM/IgG and CDC to GTKO and GTKO/HT than to WT activated PAEC. There was no significant difference in binding and CDC between activated GTKO and GTKO/HT PAEC (*$P < 0.05$, **$P < 0.01$).

significantly lower binding and CDC to GTKO and GTKO/HT PAEC than to WT PAEC ($P < 0.01$). These results indicate that (i) activation of PAEC increases antibody binding and CDC with regard to all three types of pig, and (ii) IgM/IgG binding and CDC after activation of GTKO and GTKO/HT PAEC remain significantly less than after activation of WT PAEC.

Expression of human CD46 (MCP) on cells from CD46 and GTKO/CD46 pigs

Although WT and human CD46 transgenic pigs expressed Gal on PBMC and PAEC, GTKO and GTKO/CD46 pigs and humans did not express Gal on PBMC or on aortic endothelial cells (Fig. 5A[a] and B[a]). CD46 and GTKO/CD46 and human cells expressed CD46 on their surface, but not WT and GTKO. CD46 homozygous pigs expressed higher levels of CD46 on PBMC and PAEC than GTKO/CD46 cells heterozygous for the CD46 transgene, and human cells (Fig. 5A[b] and B[b]).

Protection of pig PBMC and PAEC from CDC by the expression of human CD46

As a preliminary experiment, CDC of PBMC from CD46 pigs by both heat-inactivated (with added rabbit complement) and nonheat-inactivated human sera (in which complement activity was preserved) were compared. There was no significant difference in CDC associated with rabbit or human complement (data not shown). Because variability in endogenous complement activity might well have influenced the results if fresh sera had been used, heat-inactivated serum and rabbit complement were used in all subsequent experiments to ensure consistency. Pooled human IgM/IgG binding and CDC to PBMC and PAEC from CD46 (homozygous) and GTKO/CD46 (heterozygous for CD46) pigs were compared with WT and GTKO PBMC and PAEC. There was no difference in pooled human IgM/IgG binding to between WT and CD46, and between GTKO and GTKO/CD46 pig PBMC (Fig. 6A,B) and PAEC (Fig. 7A,B). In the CDC assay, increased resistance of CD46 and GTKO/CD46

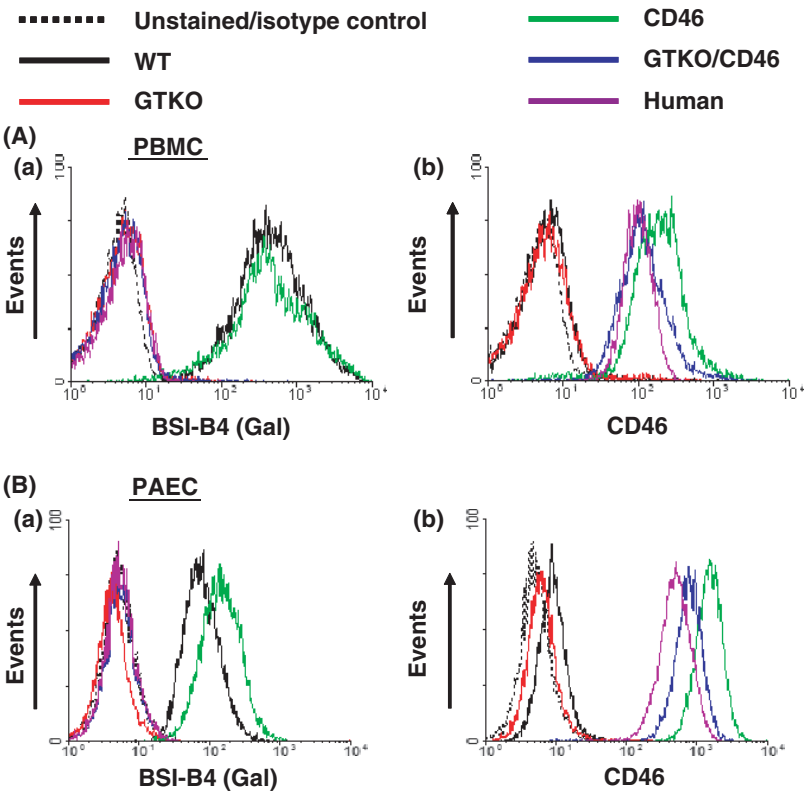

Figure 5 Expression of Gal and CD46 on PBMC (A) and aortic endothelial cells (B) from WT, GTKO, CD46 (homozygous), and GTKO/CD46 (heterozygous) pigs and humans. Cells were stained with BSI-B4 (a), or CD46 (b). Representative data of three experiments. Dotted lines – unstained or isotype control. Black lines – WT cells. Red lines – GTKO cells. Green lines – CD46 (homozygous) cells. Blue lines – GTKO/CD46 (heterozygous) cells. Purple lines – human cells. Although WT and CD46 pigs expressed Gal on PBMC and PAEC, GTKO and GTKO/CD46 pigs and humans did not express Gal on PBMC or on aortic endothelial cells (A[a] and B[a]). CD46 and GTKO/CD46 pigs expressed higher levels of CD46 on PBMC and PAEC than WT or GTKO pigs (A[b] and B[b]). CD46 (homozygous) pig PBMC and PAEC expressed higher levels of CD46 than GTKO/CD46 (heterozygous) pig cells and human cells.

PBMC and PAEC was observed compared with WT and GTKO, respectively (Figs 6C,D and 7C,D). CDC of GTKO, CD46, and GTKO/MCP PBMC was less than of WT PBMC (WT 73%, GTKO 45%, MCP 30% and GTKO/CD46 4% in 25% serum concentration, $P < 0.01$) and PAEC (WT 51%, GTKO 18%, CD46 2% and GTKO/CD46 0% in 25% serum concentration, $P < 0.01$). These results indicated that CD46 expression on PAEC leads to increased resistance against CDC. This protective effect was also demonstrated when PAEC were activated (Fig. 7D). Lysis of cells from GTKO/CD46 pigs was weak or absent when compared with the lysis of other cells, and was associated with the combination of an absence of Gal expression and the presence of CD46 expression.

Effect of CD46 expression on PAEC on lysis by sensitized baboon serum

Serum from a baboon sensitized to a GTKO pig heart was also used to further investigate whether CD46 expression on PAEC can protect against strong lysis. There was no difference in IgM and IgG binding among the four types of PAEC, except for higher binding of IgG to CD46 (homozygous) PAEC (Fig. 8A,B). Lysis of CD46 (homozygous) PAEC was significantly less than that of WT, GTKO, and GTKO/CD46 (heterozygous) PAEC [e.g. at 25% serum dilution, WT 90%; GTKO 65%; CD46 (homozygous) 12%; GTKO/CD46 (heterozygous) 33%] (all $P < 0.01$ vs. WT) (Fig. 8C,D). Lysis of CD46 (homozygous) and GTKO/CD46 (heterozygous) PAEC was less than that of WT and GTKO PAEC ($P < 0.01$).

After activation of PAEC, although there was significantly increased CDC to GTKO/CD46 (54% vs. 33%, $P < 0.05$ vs. unactivated), lysis of the other types of PAEC was increased, but not significantly [e.g. at 25% serum dilution, WT 98% vs. 90%; GTKO 76% vs. 65%; CD46 (homozygous) 19% vs. 12%] (Fig. 8D). The presence of high CD46 expression remained associated with resistance to lysis, but this was not as marked as with naive human sera.

Discussion

Organ transplantation from a WT pig into a nonimmunosuppressed primate usually results in hyperacute rejection within minutes to hours [24,25]. The primary role of anti-Gal antibodies in this response has been established [3–6]. The problem of anti-Gal antibody has been overcome by pigs homozygous for GTKO [1,2]. However, although the transplantation of a heart from a GTKO pig into an immunosuppressed nonhuman primate allows extended graft survival [7], the graft eventually fails from

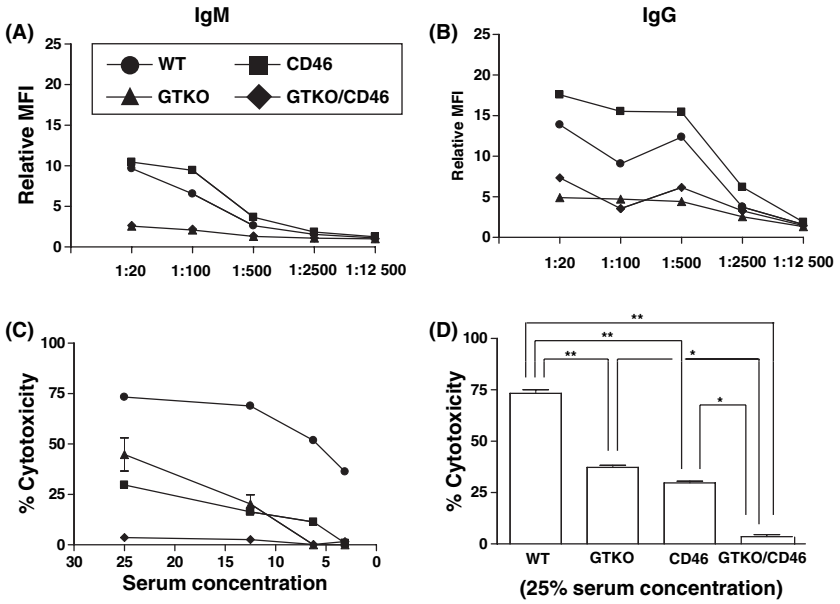

Figure 6 IgM/IgG binding and CDC of pooled human sera to WT, GTKO, CD46, and GTKO/CD46 PBMC. IgM and IgG binding to WT (●), GTKO (▲), CD46 (■), and GTKO/CD46 (◆) were measured from pooled human sera at various concentrations (1:20, 1:100, 1:500, 1:2500, and 1:12500) (A and B). Representative data of two experiments. There was no difference in pooled human IgM/IgG binding to between WT and CD46, and between GTKO and GTKO/CD46 pig PBMC, except for higher binding of IgG to CD46. Cytotoxicity by pooled human sera at various concentrations (25%, 12.5%, 6.25%, and 3.125%) against PBMC from WT (●), GTKO (▲), CD46 (■), and GTKO/CD46 (◆) was measured, and individual curves were generated (C). At 25% serum dilution, significantly lower lysis of GTKO/CD46 PBMC than that of WT, GTKO, and CD46 PBMC was observed (D). (*$P < 0.05$, **$P < 0.01$).

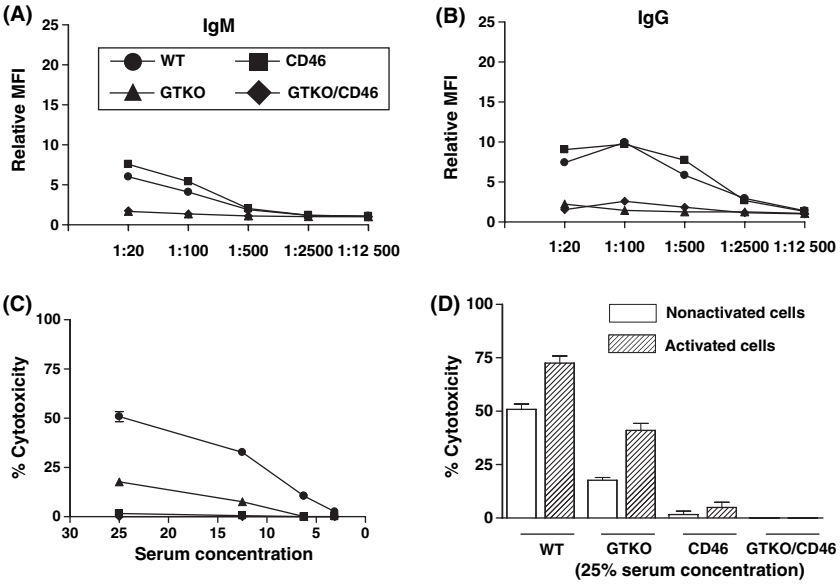

Figure 7 IgM/IgG binding and CDC of pooled human sera to WT, GTKO, CD46, and GTKO/CD46 PAEC. IgM (A) and IgG (B) binding to WT (●), GTKO (▲), CD46 (■), and GTKO/CD46 (◆) PAEC were measured using pooled human sera at various concentrations (1:20, 1:100, 1:500, 1:2500, and 1:12500). There was no difference of IgM and IgG binding between WT and CD46 PAEC or between GTKO and GTKO/CD46 PAEC. CDC of pooled human sera at various concentrations (25%, 12.5%, 6.25%, and 3.125%) against PAEC from WT (●), GTKO (▲), CD46 (■), and GTKO/CD46 (◆) pigs was measured, and individual curves were generated (C). In nonactivated PAEC (white bar), CD46 expression provided significant resistance against lysis (D). After activation of the PAEC (shaded bars), at 25% serum concentration, lysis of CD46 PAEC remained reduced or absent when compared with PAEC from the other pigs tested (D). Representative data of two experiments.

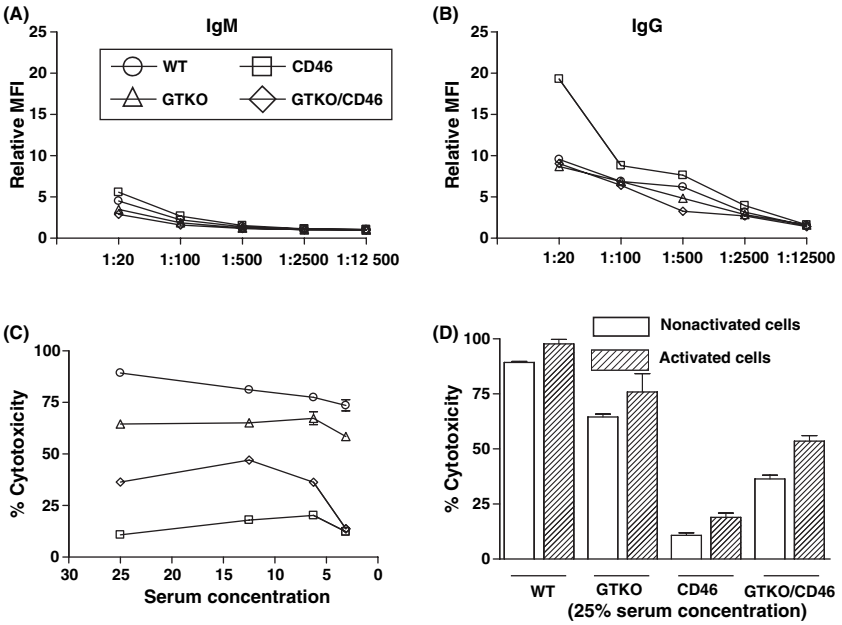

Figure 8 Protection of PAEC by the expression of human CD46 from CDC using sensitized baboon serum. IgM (A) and IgG (B) binding to WT (○), GTKO (Δ), CD46 (□), and GTKO/CD46 (◇) PAEC were measured using sensitized baboon sera at various concentrations (1:20, 1:100, 1:500, 1:2500, and 1:12500). Although a particularly high level of IgG binding to CD46 PAEC was found in sensitized baboon serum at 1:20 dilution, there was no difference in IgM/IgG binding between the other three types of pig cells. CDC of sensitized baboon serum at various concentrations (25%, 12.5%, 6.25%, and 3.125%) against PAEC from to WT (○), GTKO (Δ), CD46 (□), and GTKO/CD46 (◇) pigs was measured, and individual curves were generated (C). PAEC were also activated by IFN-γ for 48 h (D). CD46 expression, particularly in PAEC from pigs homozygous for CD46, provided significant resistance against lysis. Lysis of activated PAEC from all types of pig was increased when cells were exposed to sensitized baboon serum at 25% dilution (D). Representative data of two experiments.

a thrombotic microangiopathy that may be a form of delayed antibody-mediated rejection. The focal deposition of IgM, IgG, and C4d on the graft suggests that a component of this process involves the binding of anti-non-Gal antibodies to the vascular endothelial cells of pig. These antibodies could be either preformed or elicited.

Previous studies indicated that, whereas all healthy humans have preformed antibodies to WT PBMC, approximately one-third to one-half do not have such antibodies to GTKO PBMC [10]. Moreover, the CDC associated with anti-non-Gal antibodies is significantly weaker than that associated with anti-Gal antibodies. Whereas reactivity with WT cells results from the binding of both anti-Gal and anti-non-Gal antibodies, reactivity with GTKO cells is solely because of anti-non-Gal antibodies. GTKO PBMC are significantly less susceptible to lysis by either healthy or allosensitized sera than WT PBMC [10]. Although the absence of Gal epitopes leads to a significant reduction in both the prevalence and severity of CDC, anti-non-Gal antibodies, however, initiate a cytotoxic response which, *in vivo*, can jeopardize long-term viability of a pig organ graft [9,13]. We therefore hypothesized that protection against anti-non-Gal antibody-mediated injury might be provided by the addition of the H-transferase gene to GTKO cells, thereby increasing expression of the H(O) antigen. The expression of the H(O) antigen might cap or 'mask' non-Gal antigens such as N acetyllactosamine, that might have been exposed by GTKO and might be immunogenic.

Antibody binding and CDC of GTKO/HT PBMC and PAEC showed no differences compared with GTKO PBMC and PAEC. This observation may be associated with the considerable natural expression of the H(O) antigen on GTKO cells, particularly on PAEC (Fig. 1). With regard to protection against early antibody-mediated rejection, GTKO/HT pigs would appear to offer no advantage over GTKO pigs, although it cannot be excluded that increased expression of the H antigen might be protective against more chronic injury.

We previously used pig PBMC as target cells, whereas PAEC might provide more biologically relevant non-Gal epitopes [26,27]. The present study has demonstrated that binding and CDC to PBMC is greater than to PAEC, no matter what type of pig was the source of the cells. Binding of natural antibody to GTKO pig PBMC is generally low in naive primates [10–12], and binding to PAEC is

even lower. PBMC rather than PAEC might therefore prove preferable target cells to test antibody binding and CDC.

Although CD59 limits the formation of the terminal membrane attack complex [28], CD55 and CD46 may be the most 'useful'' transgenes for xenotransplantation. CD55 is generally considered to control the classical complement activation pathway more effectively, and CD46 the alternative activation pathway. However, CD46 is effective in protecting cells against CDC by both the classical and alternative pathways [29,30]. Its ability to control alternative pathway activation suggests that, as innate immune responses might be particularly important, it may be preferred over CD55. Alternatively, CD46 should be used in combination with CD55 [31].

The PBMC and PAEC from CD46 pigs that were used in the present study expressed higher levels of CD46 than humans and other pigs. PAEC from pigs homozygous for CD46 showed a greater resistance to CDC than did WT PAEC, and the level of resistance was equivalent to that demonstrated by GTKO and GTKO/HT PAEC, even though the binding of human IgM and IgG to PAEC from CD46 pigs was significantly higher than to GTKO and GTKO/HT PAEC, likely because of anti-Gal antibody binding to the CD46 cells. The expression of CD46 in GTKO pigs is therefore likely to provide further protection from CDC.

We found less lysis by human sera of GTKO/CD46 (heterozygous for CD46) pig PBMC and PAEC than by that of WT, GTKO, and CD46 (homozygous) cells. However, GTKO/CD46 (heterozygous) PAEC were less resistant to lysis compared with CD46 (homozygous) PAEC when serum from a baboon sensitized to non-Gal antigens (after exposure to a GTKO pig heart) was tested. This is almost certainly related to the increased expression of CD46 in the homozygous animal (Fig. 5A[b] and B[b]). A possible alternative explanation is that GTKO cells may express more non-Gal antigens than do WT cells, leading to increased antibody binding and lysis to these specific antigens that were present in greater numbers on GTKO/CD46 cells than on CD46 cells. Furthermore, the GTKO pigs were from a different founder herd than the MCP pigs; the GTKO/CD46 pigs were a mixture of these two herds. As SLA class I and II expression is likely to be different between the GTKO and CD46 strains, sensitization to a GTKO pig heart could result in a specific spectrum of anti-SLA antibodies that might be more reactive to the GTKO/CD46 (heterozygous) cells than to the CD46 (homozygous) cells. However, lysis of GTKO/CD46 PAEC by sensitized baboon serum was still less than that of GTKO PAEC. GTKO pigs homozygous for CD46 are likely to be more resistant to the effects of sensitized serum.

We have previously demonstrated that antibody binding and CDC to GTKO PBMC in naive baboon sera are similar to naïve human sera [10,11]. In the present study, binding and CDC associated with sensitized baboon serum were documented. Although expression of CD46 on GTKO pig cells only partially protected the cells from CDC caused by sensitized baboon serum, we would anticipate that this genetic combination would be significantly more protective in the presence of naive baboon serum.

Activation of PAEC (by IFN-γ) resulted in increased antibody binding and CDC of cells from all pig types. This would increase immune injury of the graft. However, GTKO, GTKO/HT, CD46, and GTKO/CD46 PAEC remained at an advantage over WT PAEC as IgM/IgG binding and CDC, although increased over nonactivated state, remained significantly less compared with WT PAEC.

Our study suggests that GTKO pigs transgenic for human CD46 are likely to be less susceptible to human serum CDC as a result of both reduced binding of antibody and increased resistance to complement-mediated injury. However, if an elicited antibody response develops, even a graft from GTKO/CD46 pig may not be protected from CDC. Our ongoing *in vivo* studies using organs from GTKO/CD46 pigs in baboons indicate that, although completely resistant to hyperacute rejection (even in the absence of agents such as cobra venom factor), the grafts develop thrombotic microangiopathy that is associated with the development of a consumptive coagulopathy in the recipient nonhuman primate (M. Ezzelarab, unpublished data). Therefore, additional genes need to be expressed in the organ-source pig. An 'anticoagulant/anti-thrombotic' gene, such as human tissue factor pathway inhibitor or CD39, is likely to be beneficial [32,33].

Authorship

HH: designed the study, performed the research, collected and analyzed the data, and wrote the paper. CL, YJL, H-CT and ME: collaborated with HH in performing the research, and have read the paper and agreed with its conclusions. DA: contributed ideas for the research and provided samples, such as blood, from the genetically modified pigs that have been generated by his group. He also contributed to the writing of the manuscript. DKC: contributed ideas for the research, and supervised the experiments and the writing of the manuscript.

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