Porcine iGb3s gene silencing provides minimal benefit for clinical xenotransplantation.

James R. Butler,[1] Nicholas J. Skill,[1] David Priestman,[2] Frances Platt ,[2] Ping Li,[1] Jose L. Estrada,[1] Gregory R. Martens,[1] Joseph M. Ladowski,[1] Matthew Tector, A.[1] Joseph Tector.[1]

[1]Department of Surgery, Indiana University School of Medicine. Indianapolis, IN USA.
[2]Department of Pharmacology, Oxford University, Oxford, UK.

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Address Correspondence to:
A. Joseph Tector MD PhD
UH 4601
550 N. University Blvd
Indianapolis IN, 46202
jtector@iupui.edu

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Abstract:

The Galα(1,3)Gal epitope (α-GAL), created by *α*-1,3-glycosyltransferase-1 (GGTA1), is a major xenoantigen causing hyper-acute rejection (HAR) in pig-to-primate and pig-to-human xenotransplantation. In response GGTA1 gene deleted pigs have been generated. However, it is unclear whether there is a residual small amount of *α*-Gal epitope expressed in GGTA1$^{-/-}$ pigs. Isoglobotrihexosylceramide synthase (iGb3s), another member of the glycosyltransferase family, catalyzes the synthesis of isoglobo-series glycosphingolipids with an α-GAL terminal disaccharide (iGb3) creating the possibility that iGb3s may be a source of α-GAL epitopes in GGTA1$^{-/-}$ animals. The objective of this study was to examine the impact of silencing the iGb3s gene (A3GalT2) on pig-to-primate and pig-to-human immune cross reactivity by creating and comparing GGTA1$^{-/-}$ pigs to GGTA1$^{-/-}$ and A3GalT2$^{-/-}$ double knockout pigs. We used the CRISPR/Cas system to target the GGTA1, and A3GalT2 genes in pigs. Both GGTA1 and A3GalT2 genes are functionally inactive in humans and baboons. CRISPR-treated cells used directly for somatic cell nuclear transfer produced single and double-gene knockout piglets in a single pregnancy. Once grown to maturity the glycoshingolipid profile (including iGb3) was assayed in renal tissue by normal phase liquid chromatography. In addition Peripheral Blood Mononuclear Cells (PBMC) were subjected to 1) comparative cross match cytotoxicity analysis against human and baboon serum and 2) IB4 staining for α-GAL/iGB3. Silencing of the iGb3s gene significantly modulated the renal glycosphingolipid profile and iGb3 was not detected. Moreover, the human and baboon serum PBMC cytotoxicity and α-GAL/iGb3 staining was unchanged by iGb3s silencing. Our data suggests that iGb3s is not a significant contributor to HAR in pig-to-primate or pig-to-human xenotransplantation.

Introduction

Galα1,3Gal (αGal) is a disaccharide synthesized in pigs but not in humans and old world monkeys. (1)(Figure 1a) Primarily, α-GAL is formed by the catalytic activity of the enzyme α-galactotransferase (GGTA1) by which galactose is added to Gal1–4GlcNAc structures present on glycoproteins or glycosphingolipids.(2) (Figure 1c) In humans the GGTA1 gene is inactive and humans develop antibodies when exposed to α-GAL epitopes.(3) Following xenotransplantation, α-GAL antibodies promote hyperacute rejection (HAR). (4) To address the issue of α-Gal epitopes, GGTA1 null animals have been generated in order to eliminate the α-GAL epitope and eliminate HAR. (5-8) However, a residual amount of α-GAL epitope reactivity has been recognized in biallelic GGTA1 knockout pig cells and implicated as a possible contributor to chronic rejection of $GGTA1^{-/-}$ organs seen in non-primate models of xenotransplantation. (9-11) One reason suggested for this is that the α-GAL epitope can be biosynthesized by other members of the galactotransferase enzyme family. (Figure 1c) In particular the enzyme isoglobotrihexosylceramide synthase (iGb3S) is known to generate the galα1,3gal disaccharides epitopes on glycosphingolipids by the addition of galactose to lactosyl ceramide. (Figure 1b) In a similar manner to GGTA1, the human genome includes iGb3s (NM001080438) and the gene is thought to be silent due to several mutations that prevent the formation of iGb3. (12).

Studies in other animals demonstrate that iGb3 levels are species specific. In rat thymus iGb3 levels are moderate whereas in mouse and human thymus iGb3 is undetectable. (13) Moreover, iGb3 is tissue specific. iGb3 is not detectable in brain, liver, kidney, spleen, thymus, testis, lung, stomach, intestine, eye, spinal cord, and plasma of mice but is detected in murine dorsal root ganglia (DRG). (13) Further studies, demonstrate that in mice iGb3 is an immune modulatory glycosphingolipid, whereby iGb3 binds to CD1d and is presented to the T-cell receptor (TCR) of invariant natural killer T (iNKT) cells. (14, 15) In addition, iGb3-primed bone marrow-derived dendritic cells exert a significant iNKT cell-mediated anti-tumor activity. (16) Consequently, both tissue- and species-specific differences in iGb3 levels make information gained from rodent models difficult to translate into the porcine-human or porcine-primate xenotransplant models. (13)

This translation is made more difficult because iGb3s gene expression does not match iGb3 epitope levels. In mice, iGb3s gene expression is ubiquitous within mouse tissues but iGb3 is detected only in the DRG. This disparity is potentially due to translational regulation or because of continuous flux in glycosphingolipid pathways. In

particular iGb3 is a substrate for iGb4 synthase which converts iGB3 to iGb4. iGb4 does not contain the terminal Galα(1,3)gal epitope.

Consequently, there is a conflict regarding the importance of iGb3/iGb3s in the literature. (12, 17) Within the context of pig-to-primate xenotransplantation, some argue for the importance of iGb3 while others have failed to detect iGb3 in porcine tissues. (18, 19) To address this we have generated iGb3s gene (A3GalT2) knockout pigs in a GGTA1$^{-/-}$ background and compared α-GAL epitope expression, antibody binding profiles, and antibody-mediated complement depended cytotoxicity between GGTA1$^{-/-}$ and GGTA1$^{-/-}$/A3GalT2$^{-/-}$ double knockout pigs.

Materials and Methods

Knockout constructs

Bicistronic CRISPR sgRNA expression vectors co-expressing the Cas9 gene were designed as described by Li et al. (20) to bind and cleave the GGTA1 gene beginning at position 293654066 of NC_010443.4 (forward: CACCGCGAAAATAATGAATGTCAA) and A3GalT2 gene beginning at position 83572809 of NCBI reference NC_010448.3 (forward: CACCGCGCTGGCAGGACGTGTCCA). Porcine liver-derived cells were cultured as previously described (21) and co-transfected with both of the above CRISPR vectors using the Neon transfection system (Life Technologies, Grand Island NY, USA) according to the manufacturer's instruction.

Selection and Genotyping

CRISPR-transfected cells were subjected to a-Gal counter-selection process, as described by Li et al., (20) to enrich for a mutation-positive population. Genomic DNA from counter-selected cells and the derivative clonal animals was obtained by GeneElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA). PCR amplification of targeted-genes was performed using primers and conditions previously described. (20)

Somatic cell nuclear transfer (SCNT)

All the animals used in this study were approved by the Institutional Biosafety and Institutional Animal Care and Use Committee of IUPUI. SCNT was performed as described by Estrada et al., (22) using in vitro matured oocytes (DeSoto Biosciences Inc, St. Seymour, TN.). Cumulus cells were removed from the oocytes by pipetting in 0.1% hyaluronidase. Only oocytes with normal morphology and a visible polar body were selected for SCNT. Oocytes were incubated in manipulation media (Ca-free NCSU-23 with 5% FBS) containing 5 µg/mL bisbenzimide and 7.5 µg/mL cytochalasin B for 15 min. Oocytes were enucleated by removing the first polar body plus metaphase II plate, and one cell was injected into each enucleated oocyte. Couples were fused and activated simultaneously by two DC pulses of 180 V for 50 µsec (BTX cell electroporator, Harvard Apparatus, Hollison, MA, USA) in 280mM Mannitol, 0. 1mM CaCl2, and 0.05mM MgCl2. Activated embryos were placed back in NCSU-23 medium with 0.4% bovine serum albumin (BSA) and cultured at 38.5 °C, 5% CO2 in a humidified atmosphere for about one

hour, before being transferred into the recipient. Recipients were synchronized occidental gilts on their first day of estrus.

αGal Phenotype analysis

Pig tissue and cells were collected in accordance with Institutional Review Board and Institutional Animal Care and Use Committee approved protocols. GGTA1-null and A3GalT2/GGTA1-null and WT PBMCs, Lymph node cells (LNC) and Splenocytes were isolated from mature animals after euthanasia. Cells were stained with isolectin GS-IB4 Alexa Fluor 488 conjugate (Life technologies, Carlsbad, CA USA) to examine the presence of the αGal epitope. Unlabeled cells were used as a negative control and WT cells served as positive control. Fluorescence detection was performed using an Accuri C6 flow cytometer (Accuri, Ann Arbor, MI, USA). Analysis of the median was performed with FlowJo version 8.8.7 (Treestar Inc., Ashland, OR).

Tissue staining and confocal microscopy

Tissues were procured from a mature A3GalT2/GGTA1-null and GGTA1-null pigs after euthanasia. Frozen sections of liver and kidney were prepared. Mounted tissues were blocked in Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE USA) in HBSS for 1 hour. The slides were then fixed in paraformaldehyde for 10 minutes. Tissues were stained with isolectin GS-IB4 Alexa Flour 488 conjugate (Life technologies, Carlsbad, CA USA) to visualize the presence of α-Gal. Tissues were incubated for 1 hour at 4^{o}C and washed three times with 0.1% HBSS Tween DAPI (Invitrogen, Carlsbad, CA) was added to all slides for 1 minute as a nuclear stain followed by two 0.1% HBSS tween washes. Tissues were mounted in ProLong Gold (Invitrogen, Carlsbad, CA). Confocal microscopy was accomplished using an Olympus FV1000 (Olympus America Inc., Center Valley PA, USA). All confocal settings were set to isotype control levels. Tissue staining of PBMC for confocal analysis was also performed as above.

Antibody binding

GGTA1-null and A3GalT2/GGTA1-null PBMCs were isolated and assessed for viability using trypan blue. Approximately 200,000 PBMCs of each group were incubated with 25% heat inactivated human sera from 10 healthy human and baboon donors for 1 hour at 4^{o}C. Samples were then washed three times with HBSS. Human IgG and IgM were detected individually with anti-human secondary antibodies conjugated to Alexa Fluor 488 (Jackson

ImmunoResearch Laboratories Inc., West Grove, PA) for 1 hour at 4°C. PBMCs were washed three times in HBSS. Fluorescence detection was performed using an Accuri C6 flow cytometer (Accuri, Ann Arbor, MI, USA). Analysis of the median was performed with FlowJo version 8.8.7 (Treestar Inc., Ashland, OR).

Complement-mediated cytotoxicity

The assay was performed as described by Diaz et al. (23) with slight modifications. In a 96-well V-bottom assay plate, 100 µl of serially diluted heat inactivated human or baboon serum was mixed with a 100 µl aliquot of PBMC from either a GGTA1-null pig or A3GalT2/GGTA1-null pig. The final concentration of PBMC in each well was 1x10^6/ml and serum concentration varied by dilution (50%, 17%, 6%, 2%, 0.6%, 0.2% and .07%). The assay was incubated for 30 mins at 4°C. After incubation, plates were centrifuged for 6 min (400x g), decanted and washed with HBSS; this step was repeated twice. Baby Rabbit complement (Cedarlane, Burlington, NC, USA) was diluted 1:15 in HBSS and 140 µl was added to each well and incubated for 30 mins at 37°C. PBMCs were labeled with a fluorescein diacetate (FDA) 1 µg/ml in acetone and propidium iodide (PI) prepared at a concentration of 50 µl/ml in phosphate-buffered saline (PBS). After incubation in complement, the samples were transferred from 96 well to flow tubes containing 100 µl HBSS and 10 µl FDA/PI. Analysis was completed by flow Cytometry. The percentage of dead cells (PI+, FDA-), damaged cells (PI+, FDA+) and live cells (PI-, FDA+) was determined by quadrant analysis. PI-/FDA- events were excluded from analysis. Background spontaneous cytotoxicity was determined by analyzing cells not exposed to human sera. Values for cytotoxicity are subsequently reported after correction by this background value using the following equation: %cytotox = (%cytotoxExp - % cytotoxSpont)/ (100 -%cytotoxSpont) where %CTXexp is the percentage of dead cells under the experimental condition.

Glycosphingolipid analysis.

Sample Collection: Porcine kidney samples were collected from GGTA1$^{-/-}$, and A3GalT2$^{-/-}$ double knock out (DKO) and wild type pigs at Indiana University Medical School, USA under IACUC approved protocol. (20, 24) Samples were flash frozen in liquid nitrogen and stored at -80°C prior to shipping under dry ice to DP at University of Oxford Department of Pharmacology, UK. **Glycosphingolipid Extraction:** Glycosphingolipids were extracted by chloroform/methanol extraction as per Svennerholm and Fredman (25) and solid phase

purification (SepPac C18) as previously described. (26) **Ceramide glycanase digestion:** Extracted and purified glycoshingolipids were digested to release carbohydrates using 50 mU ceramide glycanase at 37 °C for 18 h as previously described. (27) **Carbohydrate labeling:** The ceramide-glycanase-released oligosaccharides were labeled with anthranilic acid as previously described. (26) **Normal Phase HPLC:** Anthranilic acid labeled oligosaccharides were separated by normal phase high performance liquid chromatography and detected by fluorescence as previously described. (26)

Results

Gene knockout pigs: Porcine, liver-derived cells transfected with the bisistronic sgRNA/Cas9 knockout vectors for GGTA1 and A3GalT2 genes exhibited gene-silencing mutational events; IB4 lectin-conjugated beads facilitated the isolation of successfully mutated cells. These cells were used to create healthy clonal animals by SCNT. The genotype of animals used in this analysis exhibited an A3GalT2/GGTA1-null or a GGTA1-null background and have been previously published by Li et al.(20)

α-Gal phenotype analysis: Gal*α*3Gal epitope expression, measured by IB4 binding, was not significantly different on cells or tissue from A3GalT2/GGTA1-null or a GGTA1-null animals. At the cellular level this was true for spleen cells, lymph node cells and PBMCs (figure 2).

Tissue staining and confocal microscopy: Confocal microscopy analysis showed no visible changes to IB4 binding at the tissue level between A3GalT2/GGTA1-null and GGTA1-null animals (figure 3).

Antibody binding: IgG and IgM antibody binding from baboon sera and human sera was unchanged between PBMCs from A3GalT2/GGTA1-null and GGTA1-null animals (figure 4).

Complement-mediated cytotoxicity: Similarly, antibody-mediated complement-dependent cytotoxicity, was not reduced by the silencing of the iGB3s gene (figure 5). The lysis profiles of PBMCs from A3GalT2/GGTA1-null and GGTA1-null animals were equivalent when subjected to baboon sera and human sera.

Glycosphingolipid Analysis: Although silencing the porcine iGb3s gene did not affect patterns of IB4-binding, antibody binding, or antibody-mediated cytotoxicity, the glycosphingolipid profile of these animals was altered. HPLC analysis of tissue obtained from $GGTA1^{-/-}$, $A3GalT3^{-/-}$ double knock out (DKO) animals evidenced a unique glycosphingolipid profile compared to the wild type animals. In particular, iGb3 was not detected in renal tissue from wild type or DKO pigs. iGb3 elutes between Gb3 and GM3 and no appropriate peak was observed. (Figure 6A,B) Detection of iGb3 is difficult because of the abundance of Gb3 and GM3 in wild type pigs which limits resolution

between peaks. This is particularly challenging when GM3 is high as seen in wild type pigs (Figure 6A). In DKO pigs there was a significant reduction in α-series glycosphingolipids that include GM3 which makes detection of iGb3 easier. This reduction in GM3 is probably related to the GGTA1 gene deletion rather than deletion of A3GalT2. A similar loss of GM3 is reported in α-gal null mouse tissues. (13) Despite the reduction of GM3 detection of iGb3 was not detectable. Gb3 levels were slightly increased in DKO pigs which hindered iGb3 detection. Typically iGb3 can be detected at 1% of Gb3 levels in the absence of GM3. Because both Gb3 and GM3 were present in DKO pig kidney detection of iGb3 is significantly challenging. Our failure to detect iGb3 in wild type porcine kidneys is in accordance with previous studies of iGb3 in pigs using linear ion-trap mass spectrometry detection that was unable to detect iGb3 in pig heart, liver, kidney and pancreas. (17) In contrast to iGb3 quantification we did observe a potential reduction in the levels of iGb4 in kidneys from DKO pigs when compared to wild type controls. iGb3 is converted to iGb4 by the enzyme iGb4 synthase. iGb4 elutes between GM2 and Gb4. (Figure 6B) Moreover, iGb4 was potentially identified suggesting a rapid conversion of iGb3 to iGb4, which would explain the absence of iGb3 in wild type pig kidneys. In kidneys from DKO pigs iGb4 was not detected. This suggests the direct reduction of substrate iGb3 relating to iGb3 synthase gene deletion. (Figure 1d)

Discussion

The shortage of donor organs is the most pressing problem in clinical transplantation. (28) Xenotransplantation of pig organs is becoming an immunologically realistic solution to this problem. (24) However, to achieve clinical relevance, the major drivers of immune-mediated, antibody mediated rejection (AMR), must be understood and eliminated.(29) Over the course of evolution, humans and primates have inactivated the *α*-1,3-glycosyltransferase-1 (GGTA1) gene and form antibodies to α-GAL epitopes within xenotransplanted porcine organs. The biallelic (GGTA1) knockout pig represented the first successful step towards 'humanizing' porcine organs for xenotransplant. Nevertheless, porcine-baboon xenotransplant studies using GGTA1$^{-/-}$ pig cells have demonstrated residual α-Gal antibody formation and chronic cytotoxicity, suggesting additional sources for α-Gal epitopes. An alternative cause for α-gal biosynthesis is the enzyme isoglobotrihexosylceramide synthase (iGb3s). Some groups have suggested that iGb3s may function to replace a residual amount of α-GAL epitope in GGTA1$^{-/-}$ animals, whereas others argue against such a role. (12, 30) To address any ambiguity, this study directly investigated the role of iGb3 by creating and comparing GGTA1$^{-/-}$ pigs with GGTA1/A2GalT2$^{-/-}$ double KO pigs. Our results show that there is no meaningful contribution to α-GAL levels by iGb3s. Although there is a very small trend (figure 2) towards lower α-GAL expression in the iGb3s-null animal cells, this difference was not appreciated at the tissue level (figure 3) and both antibody binding and cytotoxicity remained unchanged by inactivating iGb3s (figure 4). We are cognizant that IB4 binding to iGb3 is reduced when compared to α-GAL and so the staining might not be a true reflection of iGb3 levels. (31) However, IB4 does bind to iGb3 and the purpose of this study was to determine comparative reductions in IB4 binding associated with iGb3s silencing. Although, the absolute levels of iGb3 are not reflective of IB4 staining there was no significant reduction in staining associated with deletion of iGb3s gene.

A lack of measured role for iGb3s in acute rejection is not unexpected. Anti α-Gal reactive antibodies are found in GGTA1 KO pigs and mice with no discernable acute or chronic reaction. (32-35) This is probably because anti α-Gal antibodies can differentiate between α-GAL carbohydrate epitopes associated with lacNAc (GGTA1) and LacCer (iGb3) core structures. (30) Most preformed antibodies recognize LacNAc form of α-GAL rather than the LacCer form, even though the terminal disaccharide is identical. Millard *et al.* do report an iGb3 specific antibody from GGTA1$^{-/-}$ mice that argues in favor of a role for iGb3s. (30) However, the specificities to antibodies produced by Milland *et al.* has been

questioned because the over expression of iGb3s is likely to generate more than just iGb3 and will generate pol-GAL glycosphingolipids such as Galα1,3Galα1,3Galα1,3Galα1,3Galβ1,4Glcβ1,1Cer. (36) Moreover, translation of murine studies to porcine-human xenotransplantation is problematic. In particular GGTA1 gene null mice are known to have lower levels of natural anti α-Gal antibodies when compared to humans and old world primates. In the study by Malland *et al.* mice were immunized against α-Gal in order to generate antibodies. (30) Consequently, it is not unexpected that the mice would generate antibodies that recognized iGb3 like epitopes. In contrast, neither humans or baboons were immunized against α-Gal, therefore antibody binding to porcine PBMN cells from GGTA1$^{-/-}$ and GGTA1$^{-/-}$/A2GalT2$^{-/-}$ pigs was minimal. Consequently, immunological quantification of iGb3 is uncertain.

In order to better understand the role of iGb3 levels in hyper acute rejection we performed glycosphingolipid analysis by NP-HPLC of kidneys from wild type and DKO pigs in order to detect iGb3. In wild type pigs iGb3 was below detectable limits. This is in accordance with previous studies of iGb3 in pigs using linear ion-trap mass spectrometry detection, whereby IGb3 was undetectable in pig heart, liver, kidney and pancreas. (17) The absence of detectable iGb3 can be explained by a rapid conversion of iGb3 to IGb4 by IGb4 synthase. (Figure 1d) In wild type pigs IGb4 was potentially detected as a peak eluting between GM2 and Gb4. Confirmation that this peak accurately represents iGb4 requires digesting the sample with a specific hexosaminidase to convert iGb4 to iGb3. For this study digestion was not performed, because the focus is on potential sources of residual α-Gal epitope and iGb4 does not contain a terminal α-gal epitope. However, by monitoring renal glycosphingolipid profiles in GGTA1 and A2GalT2 knock out pigs we did observe a general disruption of glycosphingolipid metabolism and no iGb4 peak; suggesting that deletion of iGb3 synthase reduces the iGb3 substrate for IGb4 synthase.

In conclusion, our data suggests that iGb3s is not a contributor to AMR in pig-to-primate or pig-to-human xenotransplantation. Although, iGb3s gene silencing significantly changed the renal glycosphingolipid profile, the effect on Gal*α*3Gal levels, antibody binding, and cytotoxic profiles of baboon and human sera on porcine peripheral blood mononuclearcytes (PBMCs) was neutral. In contrast, recently created triple xenoantigen KO pigs have reduced xenoreactive antibody binding and suggest that further work should continue to focus on genomic editing for the reduction of non-αGal xenoantigens and xenopeptides. (21, 24, 29, 37)

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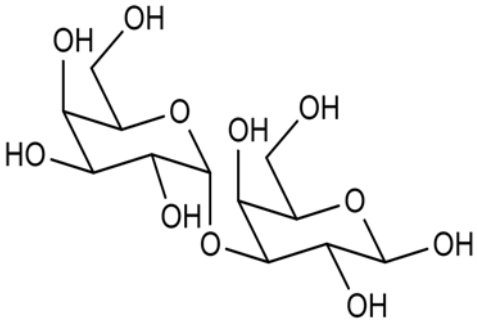
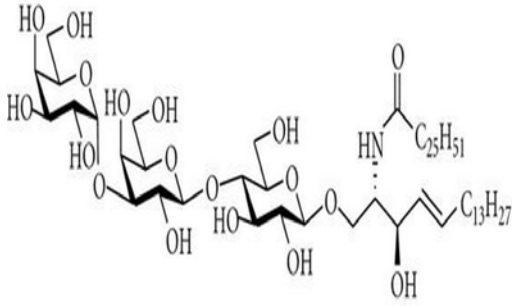
Figures and Legends:

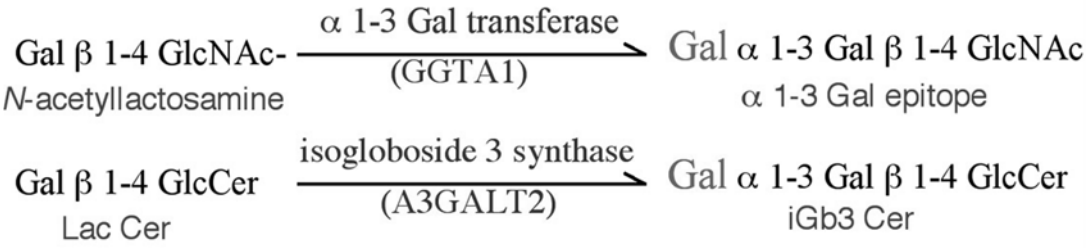
Figure 1: Illustration of alpha Gal and iGb3



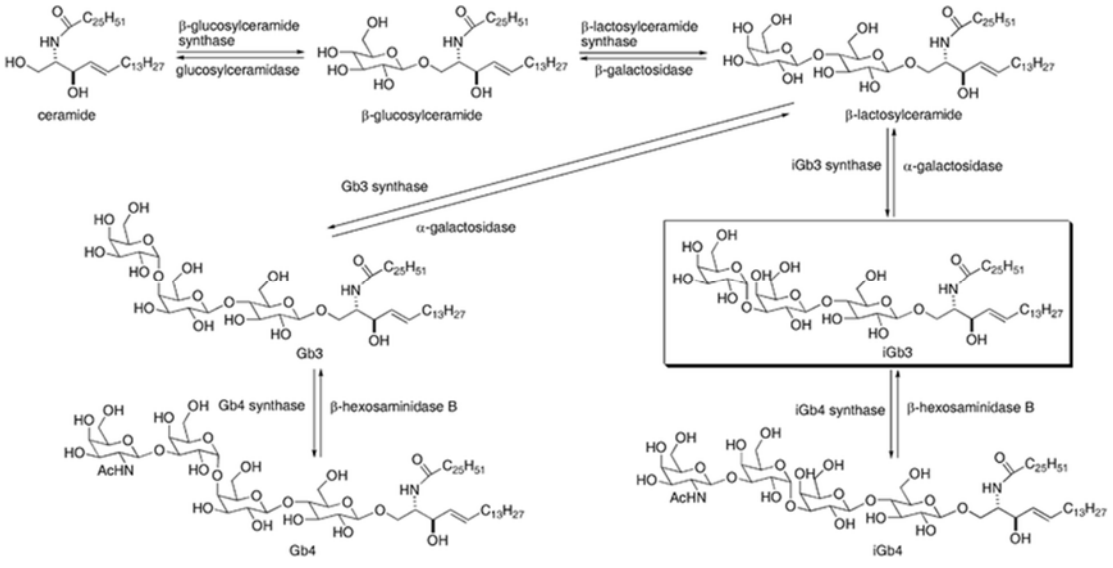
(a) Galα(1.3)Galactose



(b) Isoglobotrihexosylceramide (iGb3)



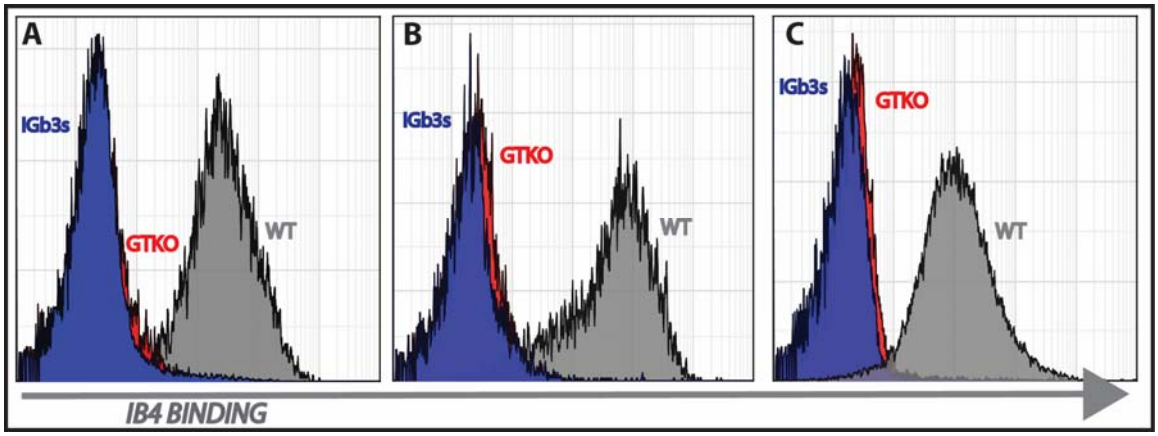
(c)



(d)

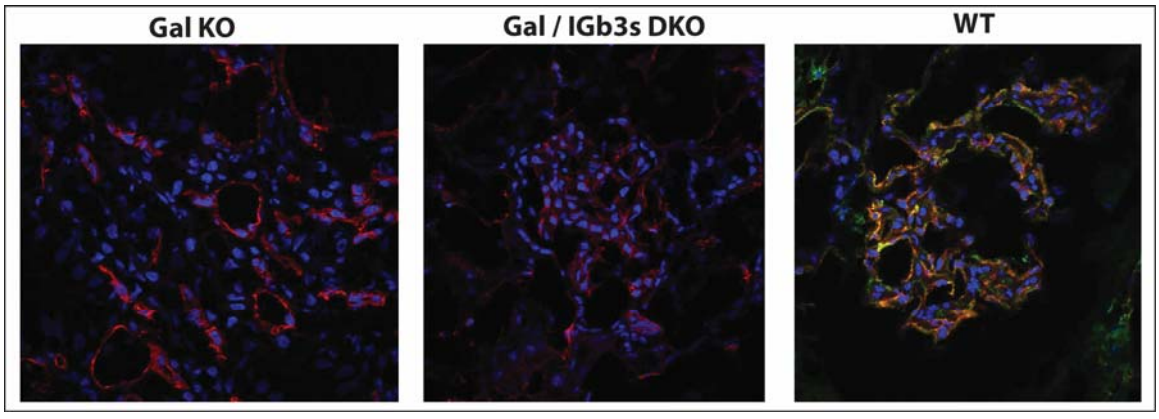
(a) Illustration of α-Gal. (b) Illustration of iGb3. (c) Comparison of α1,3 Gal transferase and iGb3 synthase (https://sites.google.com/site/abobloodtype/37-alpha-1-3-gal-nac-transferase-family). (d) Illustration of iGb3 biosynthetic pathway. (38)

Figure 2: Ib4 binding profiles of iGb3s knockout pig cells.



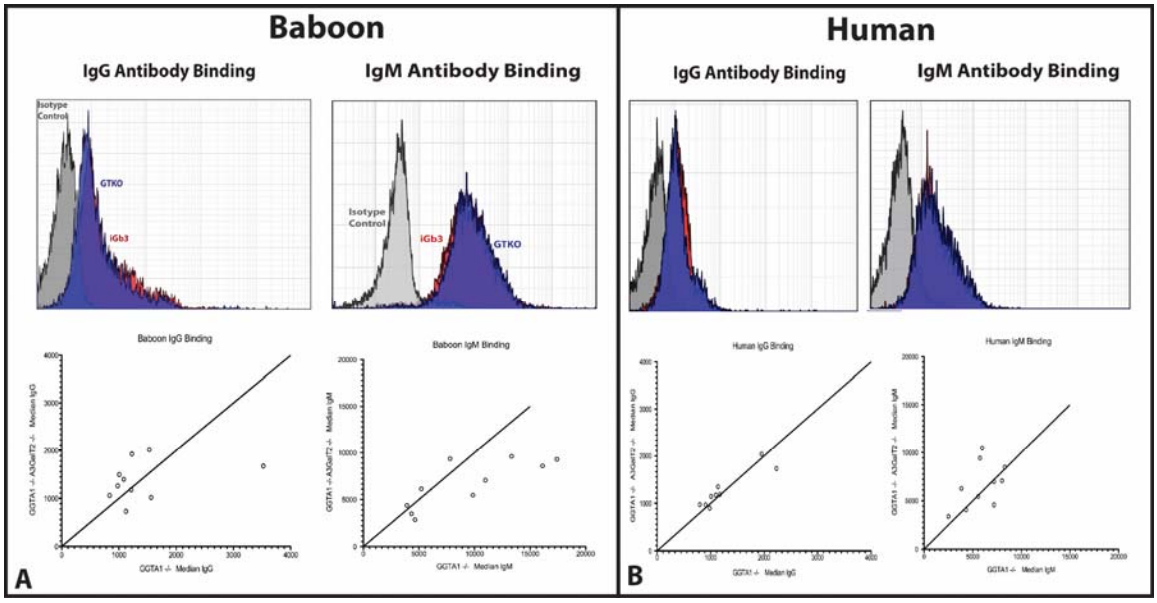
Spleen cells, Lymph node cells and PBMCs, from wild-type (WT), GGTA1-null (GTKO) or A3GalT2/GGTA1-null (iGb3) animals were incubated with a fluorescent-conjugated IB4 lectin probe to detect aGal carbohydrate profiles. For each frame, blue represents A3GalT2/GGTA1-null cells, red represents GGTA1-null cells and grey is a WT positive control. IB4 binding was not significantly affected by silencing the iGB3s gene. **A** represents IB4 binding profiles of spleen cells **B** represents IB4 binding profiles of lymph-node cells **C** represents IB4 binding profiles of PBMCs.

Figure 3: Confocal analysis of IB4 binding to iGb3s knockout pig tissues.



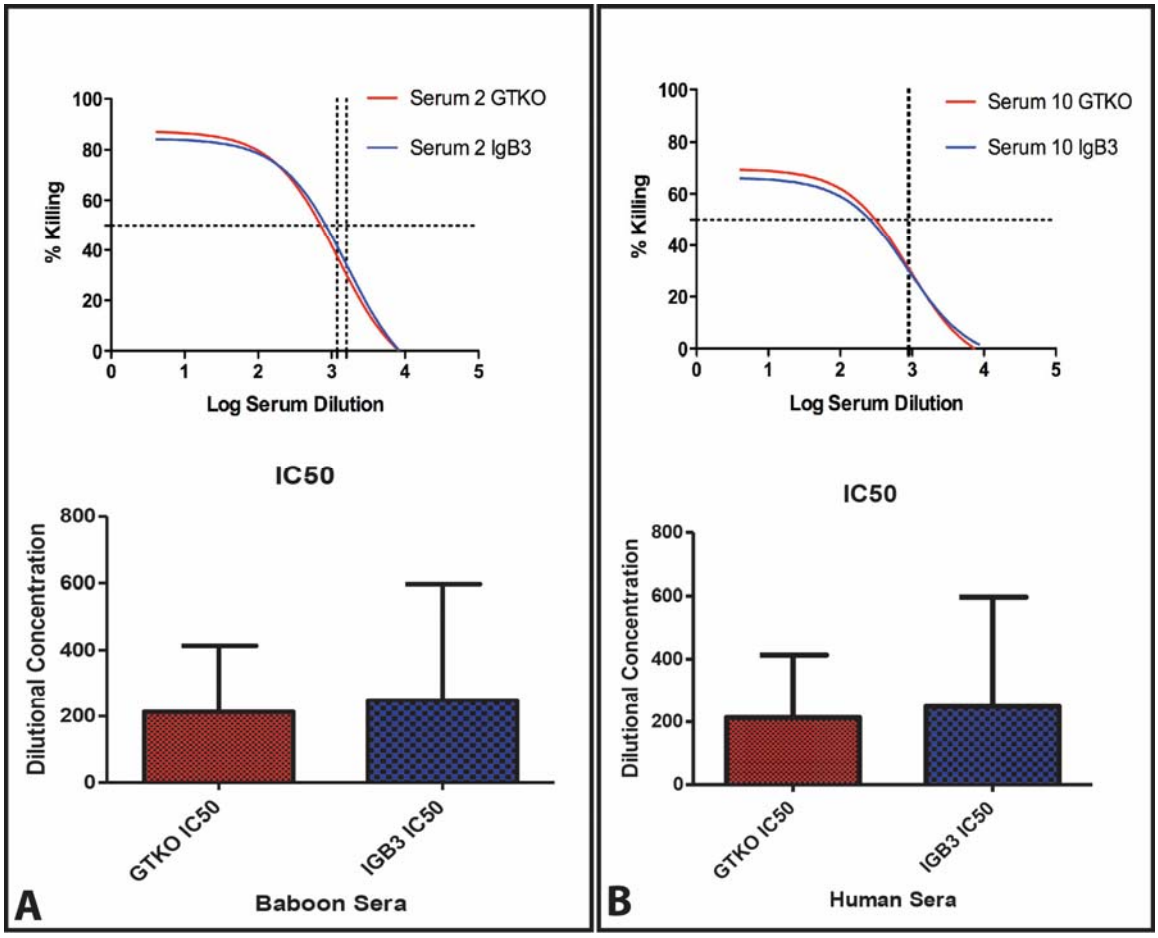
Sections of liver and kidney tissue was obtained from mature WT, GGTA1-null or A3GalT2/GGTA1-null pigs after euthanasia. These tissues were stained with IB4 lectin (green) to show Gal*α*3Gal expression level, anti-CD31 endothelial marker (25) and a DAPI (blue) nuclear stain.

Figure 4: Baboon and human antibody binding profiles of iGb3s knockout pig cells.



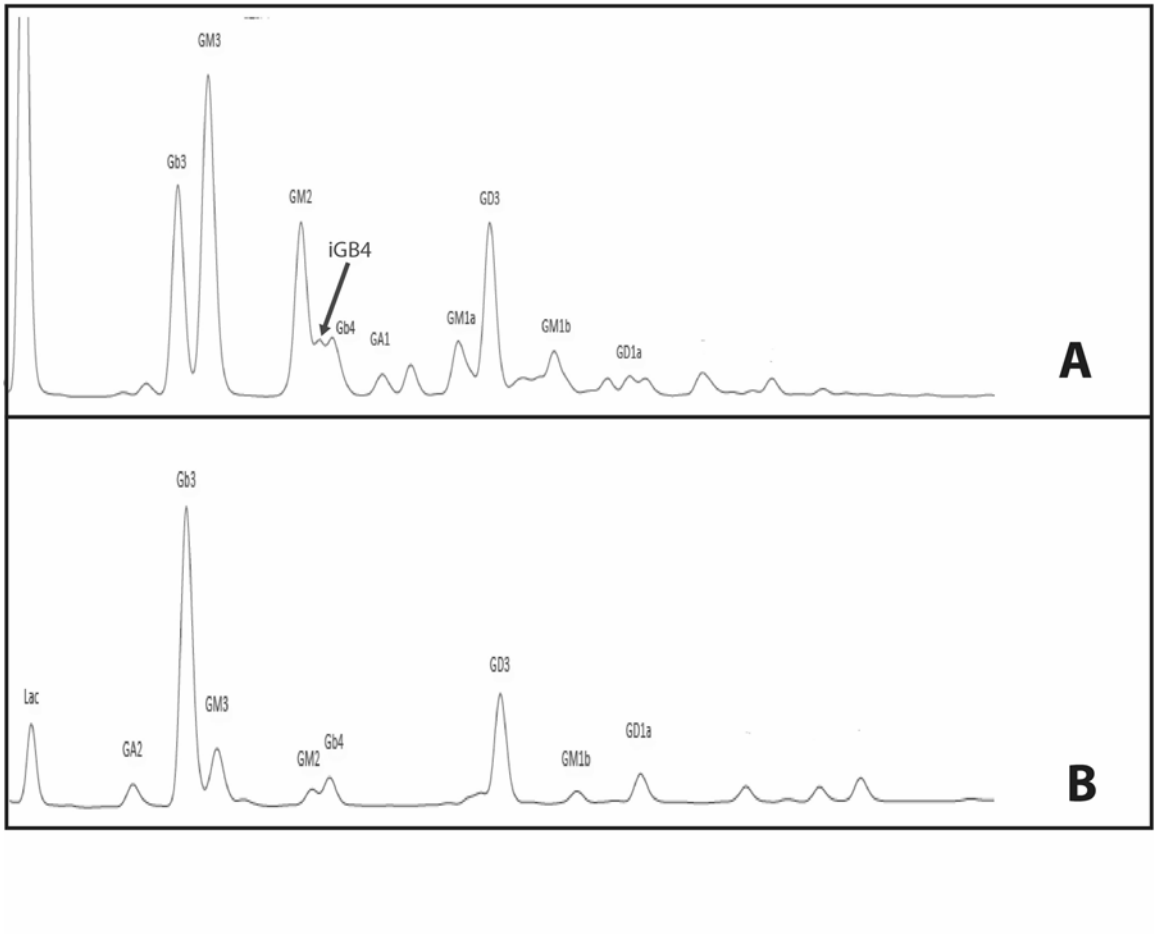
Baboon and human sera were incubated with PBMCs from swine containing inactivated GGTA1, or GGTA1 and A3GalT2 genes. Secondary fluorescent antibodies were used to detect IgG and IgM binding to the cells with median fluorescence intensity being evaluated (MFI). T-test statistical analysis was used to analyze the difference between genotype cohorts; baboon and human IgG and IgM binding was not statistically affected by silencing the iGB3s gene. For each frame, blue represents A3GalT2/GGTA1-null cells, red represents GGTA1-null cells and grey is an isotype control. **A** represents patterns of IgG and IgM binding to baboon sera. A representative histogram (above) is paired with aggregate plot of all 10 sera analyzed (below). The diagonal line indicates equivalent binding to both single and double knockout cells. **B** represents patterns of IgG and IgM binding to human sera. A representative histogram (above) is paired with aggregate plot of all 10 sera analyzed (below). The diagonal line indicates equivalent binding to both single and double knockout cells.

Figure 5: Antibody-mediated complement-dependent cytotoxicity of baboon and human sera against GGTA1-null or A3GalT2/GGTA1-null cells.



PBMCs were isolated from GGTA1-null or A3GalT2/GGTA1-null pigs. These were subjected to heat-inactivated baboon and human sera as a source of antibody. Complement-mediated cell-lysis was induced by the addition of rabbit complement and measured in a live/dead dual-labeling assay. Example curves for antibody-mediated complement-dependent cytotoxicity against GGTA1-null (25) or A3GalT2/GGTA1-null cells (blue), are above aggregate median dilutional cytotoxicity. T-test statistical analysis was used to analyze the differences between genetic cohorts; complement-mediated lysis was not statistically affected by silencing the iGB3s gene. **A** represents baboon sera toxicity **B** represents human sera toxicity.

Figure 6: Liquid chromatography analysis of kidney tissue from wild type and A3GalT2-null animals.



A represents elution patterns from WT kidney sample and **B** represents elution patterns from A3GalT2-null sample. iGb4 elutes just before Gb4 in a WT and the triplet peak in the WT pig becomes a doublet in the A3GalT2- null sample. This is indicative of upstream silencing of the iGb3s enzyme.