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## Reduced binding of human antibodies to cells from GGTA1/ CMAH knockout pigs

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### Abstract

Xenotransplantation using genetically modified pig organs could solve the donor organ shortage problem. Two inactivated genes that make humans unique from pigs are GGTA1 and CMAH, the products of which produce the carbohydrate epitopes, aGal and Neu5Gc that attract preformed human antibody. When the GGTA1 and CMAH genes were deleted in pigs human antibody binding was reduced in preliminary analysis. We analyzed the binding of human IgM and IgG from 121 healthy human serum samples for binding to GGTA1 KO and GGTA1/CMAH KO peripheral blood mononuclear cells (PBMC). We analyzed a sub population for reactivity towards genetically modified pig PBMC as compared to chimpanzee and human PBMC. Deletion of the GGTA1 and CMAH genes in pigs improved the crossmatch results beyond those observed with chimpanzees. Sorting the 121 human samples tested against the GGTA1/CMAH KO pig PBMC did not reveal a distinguishing feature such as blood group, age or gender. Modification of genes to make pig carbohydrates more similar to humans has improved the cross match with human serum significantly.

### Keywords

Xenotransplantation; crossmatch; antibody-mediated rejection; genetically modified pigs

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The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

## Introduction

Solid organ transplantation has developed into a field where patients' lives are transformed from the brink of death to near normal. Improvements have been made in nearly all aspects of the field except one, the availability of donor organs. Xenotransplantation could solve the organ shortage problem, but has been limited because of the antibody barrier posed by xenoantigens present on the surface of all pig organs (1, 2).

In 1964 Reemtsma and Starzl published a series of non-human primate to human renal xenotransplants. Reemtsma used chimpanzee kidneys in six patients who survived 23 days to 9 months post-transplant (3). Starzl transplanted baboon kidneys into six patients who survived 10–60 days post-transplant (4). Immunosuppression in both series consisted of azathioprine, corticosteroids, and mitomycin C. Since both series were performed prior to the understanding that antibodies were responsible for hyperacute rejection, there were no detailed anti-donor antibody studies available (5).

Despite the advances in immunosuppression, clinical xenotransplantation did not progress, and the use of primates as donors fell out of favor. The use of pigs as organ donors became the focus of xenotransplantation, because pigs are plentiful, physiologically similar to humans, and less likely than primates to transmit zoonotic viruses (6). Hyperacute rejection was the survival-limiting barrier of pig-to-human xenografts, caused by preexisting xenoreactive antibodies and complement activation within the graft (7). Galactose  $\alpha$ -1,3 galactose (aGal) was identified as a major xenoantigen to which xenoreactive antibodies bound and fixed complement (8). The development of somatic cell nuclear transfer (SCNT) and genetic engineering made it possible to create galactosyltransferase knockout (GGTA1 KO) pigs, whose organs were not hyperacutely rejected when transplanted into immunosuppressed baboons (9). Tolerogenic immunosuppressive protocols resulted in longer survival, but preformed and de novo xenoreactive antibodies remained a barrier to further xenograft survival (9, 10).

The expression of another carbohydrate xenoantigen N-glycolylneuraminic acid (Neu5Gc) has been eliminated in addition to aGal. Neu5Gc is present in pigs, but not in humans because like the GGTA1 gene, the CMAH gene was inactivated during the course of evolution (11–14). Our initial characterization of the aGal/Neu5Gc deficient pig has indicated that Neu5Gc is a significant xenoantigen present in all people we have tested thus far (14). Neu5Gc is present in all primates, and as a result the non-human primate is not a suitable model with which to test these new pig organs.

The work described in this report evaluates three issues regarding the GGTA1/CMAH KO pig; 1) the proportion of people for whom the GGTA1/CMAH KO has an improved crossmatch compared to the GGTA1 KO pig, 2) a comparison of the degree of discordance of the GGTA1/CMAH KO pig, GGTA1 KO pig and chimpanzees with regards to xenoreactive antibody levels present in human serum, and 3) whether there are patients who have lower or higher levels of remaining xenoreactive antibodies with regards to; blood type, age or gender.

## Materials and Methods

### Serum antibody binding to GGTA1-KO and double-KO PBMCs (Flow Crossmatch)

Blood samples were collected from healthy humans or cloned genetically modified pigs (blood type O) using Institutional Review Board and Institutional Animal Care and Use Committee approved protocols (IRB#1110007111 and IACUC#10447). The 121 healthy human serum samples were collected from an FDA registered center using protocols approved by the American Association of Blood Banks (Valley Biomedical, Winchester, VA and Sanguine Biosciences Inc., Valencia, CA). Blood from 3 chimpanzees was obtained from the Southwest National Primate Research Center (Texas Biomedical Research Institute, San Antonio, TX). Blood from cloned GGTA1/CMAH knockout pigs (type O), human (type A), and chimpanzees (two blood type A and one undetermined) corresponding to Figure 2 were separated using Ficoll-Paque Plus to collect PBMCs. GGTA1 KO, GGTA1/CMAH KO, chimpanzee and human PBMC were incubated with 25% heat inactivated serum from 5 human subjects: human 1 (type O), human 2 (type A), human 3 (type A), human 4 (type O), human 5 (type O). PBMC were stained with DyLight 488-conjugated Donkey anti-Human IgM or, DyLight 488 Goat anti-Human IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Flow cytometry was performed using an Accuri C6 flow cytometer (Accuri, Ann Arbor, MI, USA). Antibody binding data to all types of PBMC within each experiment were collected at the same time. Human serum antibody binding was analyzed using FlowJo version 8.8.7 (Treestar Inc. Ashland, OR). The forward and side scatter gate excluded only dead cells and debris. Histograms of the fluorescent channel were used for determination of MFI and overlay. Flow cytometric results were reported as averages of the Median FI values where P values were determined by unpaired two-tailed T test shown for human IgM or IgG binding to GGTA1/CMAH KO PBMC (Figure 3).

## Results

### Impact of CMAH deletion on human crossmatch with GGTA1 KO PBMC

Cells from the GGTA1/CMAH KO pig bound less human antibody than a GGTA1 KO pig when tested against a small population of human sera, yet showed some variability (14). To more clearly define the benefit of the GGTA1/CMAH KO pig cells among a larger human population we tested 121 human serum samples for IgM and IgG binding to genetically modified pig PBMC. Human IgM and IgG binding to GGTA1 KO and GGTA1/CMAH KO PBMC analyzed by scatter plot (Figure 1) indicated that mutating the CMAH gene in addition to GGTA1 reduced antibody binding for 117 of the 121 individual tested (Figure 1 shown below the trend line). Pearson correlation analysis was calculated for IgM and IgG binding to GGTA1 KO and GGTA1/CMAH KO PBMC ( $r=0.157$ , 95% CI  $-0.0225$  to  $0.326$  and  $r=0.257$ , 95% CI  $0.081$  to  $0.416$ , respectively) suggested a non-linear relationship where  $-1$  is a perfect negative linear,  $0$  is non-linear and  $1$  is a perfect linear relationship.

## Crossmatch of GGTA1/CMAH pig with human serum compares favorably to the chimpanzee

Chimpanzee renal xenografts were rejected less vigorously than baboon kidneys (3, 4). We compared IgM and IgG binding from 5 human serum samples to PBMCs from 3 GGTA1/CMAH pigs, 3 chimpanzees and 3 humans to evaluate whether we had created a concordant crossmatch pig. In 4 of the 5 human serum samples the GGTA1/CMAH KO pig PBMC bound less IgM and IgG than chimpanzee PBMC (Figure 2). The similarity of replicates suggests that differences between A and O blood groups did not influence human antibody binding. The degree of concordance or discordance was determined by comparison to the human antibody binding of human PBMC.

## Applicability of CMAH benefit to segments of the human population

We evaluated whether human reactivity to the GGTA1/CMAH KO pigs was broadly applicable across blood types A (n=38), AB (n=22), B (n=30), O (n=31), age defined as old (n=59): 50–65 years or young (n=62): 18–30 years and gender: female (n=57) and male (n=64) (Figure 3). There were small but significant differences in IgG binding between young and old (P=0.001) and IgG binding to the O blood group as compared to A (P<0.001), AB (P<0.001) and B (P=0.008). Three individuals from the blood group O population had 10–20 times the antibody binding of the other human samples tested. When these 3 individuals were excluded, there was no difference in human reactivity to GGTA1/CMAH KO PBMC based on blood group. Despite these small variations, the majority of individuals bound similar amounts of IgG and IgM. When Human IgG binding was compared between blood groups A and AB (P=0.932), A and B (P=0.892) or AB and B (P=0.06) no significant differences were found. Human IgM binding to all blood groups was not significantly different (A and O P=0.056, AB and O P=0.147, B and O P=0.075, A and AB P=0.966, A and B P=0.741, and AB and B P=0.76). There was no difference in IgG or IgM binding to male or female (P=0.172 and P=0.46, respectively). There was no difference in human IgM binding to the young or old (P=0.919).

## Discussion

Despite tremendous improvements in solid organ transplantation in the past 40 years, xenotransplantation has remained limited to the experimental realm largely because of the risk of antibody-mediated rejection (AMR). The creation of the GGTA1 KO pig in 2002 was a major advance for xenotransplantation, but AMR remained the cause of graft failure in primate models (9, 15, 16). New pigs with reduced xenoantigenicity were not created because of the difficulty and expense associated with the creation of knockout pigs. The development of nuclease based genome editing made it possible to knockout more than one gene at a time in five months (14, 17, 18). We used ZFN technology to create the GGTA1/CMAH KO pig, removing aGal and Neu5Gc from the surface of the pig cells (14). To evaluate the changes in immunoreactivity of the genetically modified pigs we used the clinical crossmatch test where donor PBMC bound by recipient serum antibodies is an predictor of graft success. Our data show that the crossmatch with the GGTA1/CMAH KO pig is an improvement when compared to the GGTA1 KO pig for nearly all patients tested. Our previous characterization of these genetically modified pigs suggested that the heart,

kidney and liver reflect the same loss of aGal and Neu5Gc as measured on PBMC (14). Therefore the crossmatch analysis is likely a good indicator of the changes in recipient immunoreactivity to the donor organ. It is possible, however, that new carbohydrate structures or organ specific glycosylations may become apparent xenoantigens as we reduce overall antibody binding.

Reemstma's chimpanzee and Starzl's baboon xenograft experiences showed that the non-human primate donor kidney could be used without the threat of hyperacute rejection (19, 20). Their clinical series were performed prior to the discovery that antibodies were responsible for aggressive forms of transplant rejection, and so their clinical series did not include detailed antibody data in the form of crossmatch analysis (5). Calne suggested that the first step in moving toward pig-to-human xenotransplantation was to convert the rejection reaction from discordant to concordant (5). Our crossmatch data show that humans in 4 of the 5 cases tested, have less antibody binding to GGTA1/CMAH KO pig than chimpanzee PBMCs. The data suggests that the GGTA1/CMAH KO pig and the chimpanzee are similar in terms of their discordance. This finding is significant in light of improvements in the diagnosis and management of sensitized patients on the waitlist. It may now make sense to re-evaluate IVIG for its ability to improve the crossmatch of human serum with the new GGTA1/CMAH KO pig. If IVIG or plasmapheresis can remove the remaining xenoantibodies in human serum, it is possible that a clinically used desensitization protocol and modern immunosuppression could pave the way for a clinical trial of pig-to-human renal xenografts (21). Baboons have a functional CMAH enzyme and express Neu5Gc. Baboon serum crossmatches with GGTA1/CMAH KO PBMCs is significantly worse than for GGTA1 KO PBMCs (data not shown), making it unlikely that these new pigs can be tested in a primate model. Thrombotic microangiopathy is the final common pathway for GGTA1 KO xenograft failure in baboons, raising the question of whether immunologic injury, or species thromboregulatory incompatibility is the next barrier to moving forward with clinical xenotransplantation (22). McGregor's group showed increased immunosuppression, and not anticoagulation, improved heterotopic GGTA1 KO pig heart survival in baboons (23). Their work suggests that there is more to be gained from the continuing to eliminate xenoantigens in pigs.

Gene editing technology is improving at an incredible rate, and with the advent of the new Cas9 guide RNA technology, making new modified pigs has become simpler, faster, and less expensive than ever (24). Taking advantage of these advances could allow evaluation of other identified xenoantigens for improvement in the human serum crossmatch through additional genetic modifications in pigs. The iGb3 synthase adds aGal to glycolipids, and studies with GGTA1 KO fibroblasts showed that there is still gal present on these cells at 1–2% of wild type levels (25). Studies in GGTA1 KO pigs have failed to show the presence of gal in organs, but studies in GGTA1 KO mice have shown that gal is still present despite the GGTA1 deletion (26–28). Creation of an iGb3S KO pig is the simplest way to definitively evaluate glycolipid bound gal $\alpha$ (1,3)gal as a xenoantigen. We are currently evaluating GGTA1/iGb3S and GGTA1/iGb3S/CMAH KO pigs in our lab.

Our data also suggests that the crossmatch benefit of the CMAH deletion is widespread in the human population and is not limited to a particular blood type, age or gender. People

with blood type O did have increased antibody binding to the GGTA1/CMAH KO pigs when compared to people of blood groups A, B, and AB. The increased antibody binding in blood group individuals was the result of a small fraction of outliers, and while statistically significant, for most individuals their antibody binding was not significantly different from those on the other blood groups.

In summary the creation of the GGTA1/CMAH KO pig is an improvement over the GGTA1 KO pig as far as reducing the antibody barrier to xenotransplantation is concerned. The GGTA1/CMAH KO pig was similar to the chimpanzee with regards to degree of discordance. Testing with desensitization protocols will determine whether the remaining xenoantibody can be managed with IVIG or plasmapheresis to facilitate clinical application of xenotransplantation. This work also highlights the fact that further reduction in the xenoantibody barrier may be achieved through the deletion of additional xenoantigens in donor pigs.

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## ABBREVIATIONS

<b>aGal</b>	galactose alpha-1,3 galactose
<b>neu5Gc</b>	N-glycolyl neuraminic acid
<b>neu5Ac</b>	N-acetyl neuraminic acid
<b>CMAH</b>	cytidine monophosphate-N-acetylneuraminic acid hydroxylase
<b>PBMCs</b>	peripheral blood mononuclear cells
<b>HBSS</b>	Hank's balanced salt solution

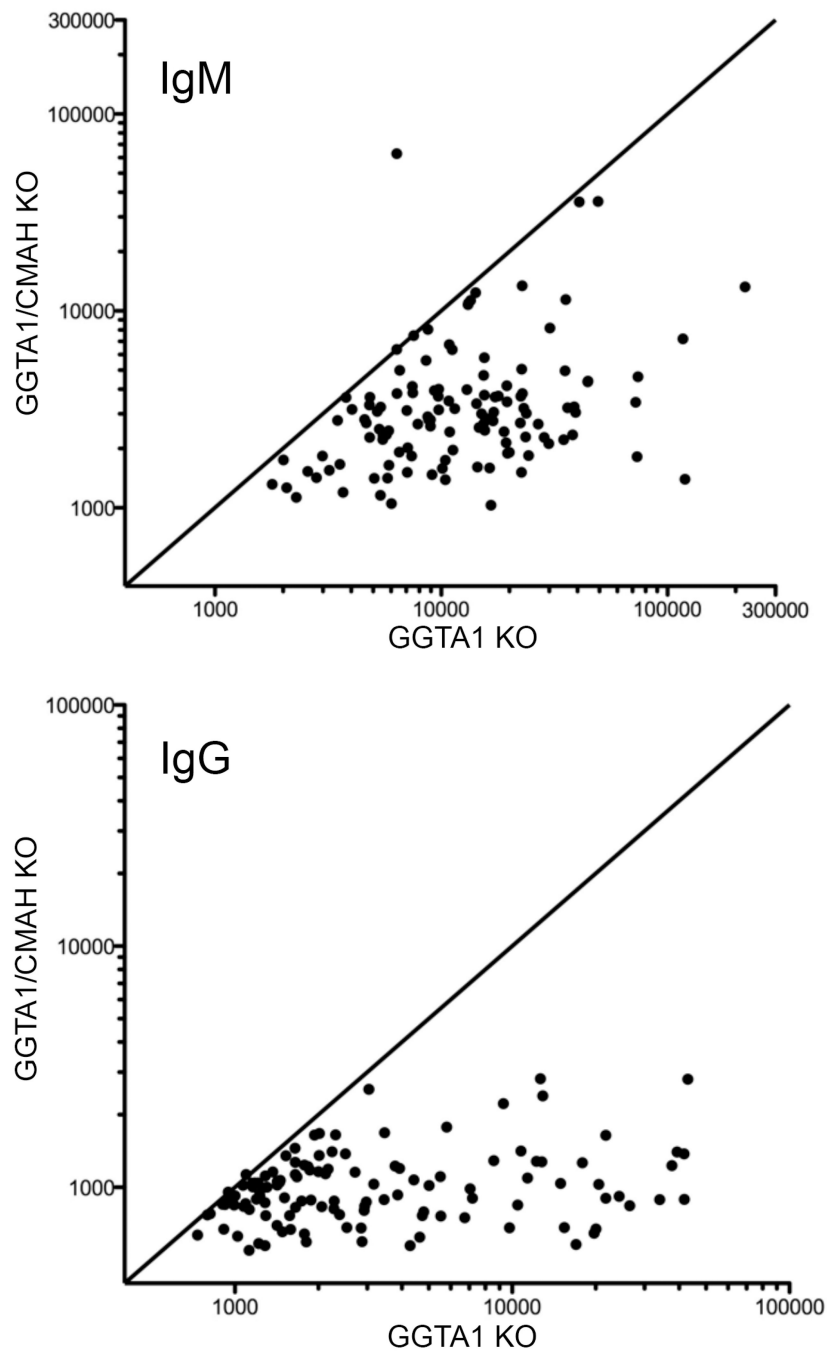
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**Figure 1.** Scatter plot of human IgM or IgG binding to either PBMC from GGTA1 KO (x-axis) and GGTA1/CMAH KO (y-axis) pigs. Values falling below the trend line indicates less antibody binding to the GGTA1/CMAH KO PBMC than to the GGTA1 KO PBMC.



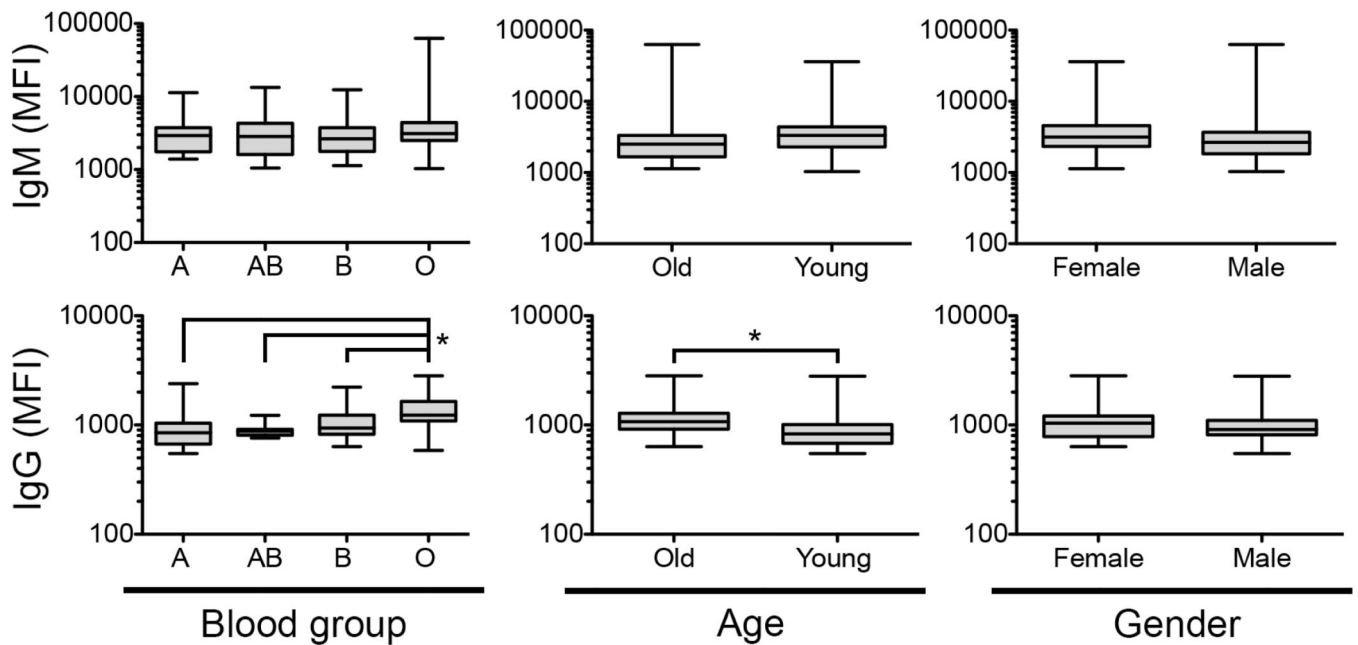
antibodies in the absence of human serum are shown to indicate background fluorescence (2° only).

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**Figure 3.**

Human IgM or IgG binding to GGTA1/CMAH KO PBMC sorted by blood types A (n=38), AB (n=22), B (n=30), O (n=31), age defined as young (n=62): 18–30 years or old (n=59): 50–65 years, and gender: female (n=57) and male (n=64). Box and whisker plots are shown with the maximum and minimum values. Statistical significance was determined by unpaired two-tailed T test and indicated with an asterisk (\*).