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An approach to induction of tolerance to pig cardiac xenografts in neonates

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Abstract

There is a continuing need for donor hearts for infants with complex congenital heart defects. The transplantation of hearts from neonatal pigs would be an alternative to human organs, particularly if donor-specific immunological tolerance could be achieved. The great majority of infant humans do not make natural (preformed) antibodies against triple-knockout (TKO) pigs (that do not express any of the three known pig antigens against which humans have natural anti-pig antibodies). The transplantation of a heart from a TKO pig into an infant would therefore minimize any risk of early antibody-mediated rejection, and, with adequate immunosuppressive therapy, prolonged graft survival may well be achieved. Total host thymectomy (commonly carried out at the time of orthotopic heart transplantation in this age group) ± residual T-cell depletion and donor-specific pig thymus tissue transplantation might induce T-cell tolerance and allow immunosuppressive therapy to be discontinued (if there is in vitro evidence of T-cell and B-cell nonresponsiveness to donor-specific pig cells). Even if tolerance were not achieved, with continuing immunosuppressive therapy, the graft would likely “bridge” the patient until a suitable allograft became available or be associated with prolonged xenograft function.

Keywords

heart; infant; neonate; pig; tolerance; xenotransplantation

1 | INTRODUCTION

A considerable experimental effort has been made to resolve the problem of the shortage of organs from human donors for clinical transplantation by the transplantation of organs from genetically engineered pigs into nonhuman primates (NHPs).[1] Survival of genetically engineered pig heterotopic (non-life-supporting) hearts and life-supporting kidneys is now

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CONFLICT OF INTEREST

None declared.

being measured in months or even years.[2–4] These encouraging results have been obtained largely by two key genetic approaches–(a) deletion of pig antigens against which humans (and NHPs) have natural (preformed) antibodies[5,6] and (b) the introduction into the pig of transgenes for human complement- and/or coagulation-regulatory proteins.[1]

There is a particular lack of deceased human donor hearts for transplantation in neonates and infants with life-threatening, complex congenital cardiac defects, for example, hypoplastic left heart syndrome, or congenital cardiomyopathy. Conventional management of these patients is less than ideal, and the outcome is certainly not as good as after heart allotransplantation.[7] Furthermore, in this age group, mechanical assist devices are less than optimal.

With regard to heart transplantation, at Loma Linda University Children's Hospital, heart transplants in neonates are associated with an almost 60% 25-years' survival,[7] and more recent data indicate patient survival at 1, 5, 10, and 15 years is currently 88%, 81%, 75%, and 69%, respectively (Bailey LL, and Fitts J, personal communication). Everitt et al[8] reported 1, 5, and 10 years' survivals of 79%, 77%, and 65% for unpalliated hypoplastic left heart syndrome, and the ISHLT reported a median survival of 22.3 years for all infants who underwent heart transplantation.[9] Early mortality is probably associated with deterioration in the patient's health status during the wait for a human donor heart (which is a mean of >2 months, but can extend to >1 year[10]). (This would be resolved if a heart was available immediately after birth.)

The difficulty in obtaining suitable donor hearts has resulted in a preference for staged palliation as primary therapy,[11] which usually involves three operations during the first 3-6 years of life. The results remain suboptimal, with a 60%-70% 5-year survival, even though some patients may not have completed the course of palliative procedures. Even when staged palliation is complete, the quality of life of the patient may remain impaired.[12] As the patient is left with a systemic right ventricle, heart transplantation may still be required, which may be complicated because some patients become allosensitized.[13] At this stage, heart transplantation is associated with relatively poor 1-year survival.[14] There is, therefore, a cumulative mortality and morbidity after each procedure.

The ready availability of a pig heart may allow the transplant to be carried out immediately after birth, thus avoiding any deterioration in health. The diagnosis of a complex cardiac abnormality can often be made during fetal life, and so the heart transplant can be planned in advance. Although we do not believe that the induction of tolerance is essential for the success of a cardiac xenograft, it would certainly be an advantage, particularly in infants and children in whom the graft would ideally need to function throughout life.

The concept of neonatal tolerance (in which the recipient's immune system makes no effort to reject the graft) is not new, but the ability to achieve T-cell and B-cell tolerance to either an ABO-incompatible allograft or a genetically engineered pig xenograft has not been fully explored even in a clinically relevant neonatal NHP model. The extensive clinical studies by West and her colleagues have demonstrated that an ABO-incompatible heart graft in infants is associated with the development of B-cell tolerance to the incompatible A or B

antigen.[15–17] These specific antibodies are either no longer produced by the recipient or a state of "accommodation" is achieved.[16,18] It is not known whether T-cell tolerance can also be achieved. The potential to develop donor-specific tolerance during the first few months of life would be of immense importance in heart transplantation in infants.

We suggest that the induction of tolerance should be explored by a combination of (a) genetically engineered pigs as the sources of the organs, (b) novel costimulation blockade-based immunosuppressive therapy, (c) thymectomy, and (d) donor-specific pig thymic transplantation.

2 | DEVELOPMENT OF THE IMMUNE SYSTEM IN NEONATES

The immune system of neonatal humans and NHPs is "immature" and relatively easily "manipulated,"[19] but it is not known whether B-cell and/or T-cell tolerance to a xenograft can be achieved.

Neonatal baboons do not hyperacutely reject pig heart grafts;[20–22] this is associated with an absence of preformed "natural" antibodies in baboons (and humans) at that age.[21–24] From previous studies by our group, infant baboons and humans begin to develop natural anti-AB blood group and anti-pig antibodies after approximately 3 months of age (Figure 1).[24,25] In infant baboons, the mixed lymphocyte reaction (MLR) against baboon peripheral blood mononuclear cells (PBMCs) or pig PBMCs is developed, but relatively weak.[25,26] There is, therefore, a "window of opportunity" during which organ transplantation could be carried out in the absence of preformed antibodies and in the presence of a "malleable" T-cell proliferative response.

However, if an AB-incompatible artery patch allograft or a pig xenograft is transplanted in a baboon at 3 months without immunosuppressive therapy, a donor-specific antibody response develops within 2 weeks.[25] In contrast, when the graft is protected by a regimen of T-cell costimulation pathway blockade (anti-CD154 monoclonal antibody [mAb]) plus mycophenolate mofetil (MMF), no antibodies develop to the specific A or B or pig antigens expressed on the graft (or, of course, to self A or B antigens).[25] One baboon with an AB-incompatible allograft developed neither anti-AB antibodies nor any increase on MLR during the 4 months' period of follow-up after discontinuation of costimulation blockade therapy, suggesting that both B-cell and T-cell tolerance might have been induced.

Furthermore, when treatment with anti-CD154mAb alone was initiated at 1 month and continued for 6 months (in the *absence* of an allograft or xenograft), the baboon did not develop any anti-A or anti-B or anti-pig antibodies until 4 months after discontinuation of therapy, that is, at 11 months of age.

These data strongly suggest that (a) natural antibodies directed to carbohydrate antigens may be at least partly T cell-dependent, and (b) both T-cell and B-cell responses can remain suppressed by costimulation blockade therapy even in the presence of an AB-incompatible allograft or pig xenograft.

Anti-CD154mAbs may not be available for clinical transplantation within the near future in view of their thrombogenic properties, but an anti-CD40mAb may provide an alternative. An anti-CD40mAb (2C10R4) has proven effective in preventing T cell and elicited antibody responses to a genetically engineered pig organ xenograft in adult NHPs for months or even years.[2,4,27] We believe that the effect of costimulation blockade on the indirect pathway of T-cell activation is particularly important.

Relatively recently, pigs have been produced that do not express the three known antigens against which humans have natural antibodies, that is, triple-knockout (TKO) pigs.[5] These pigs do not express galactose-α1,3-galactose (Gal), N-glycolylneuraminic acid (Neu5Gc), or Sda (a product of β4GalNT2-KO) (Figure 2).[5] We have measured antibody binding to TKO pig red blood cells (that do not express swine leukocyte antigens [SLA]) in the sera of 50 human neonates and infants, and determined that there is no IgM or IgG binding in the large majority of cases (48/50) (Hara, et al, unpublished data). As many adults do not have antibodies directed against TKO pig cells, in our study, we did not identify any maternally derived cross-placental IgG in these neonates. Furthermore, almost one-third of *adult* patients on the wait-list for a kidney allograft have no detectable anti-pig antibodies to TKO pig cells.[6]

These data indicate that a TKO pig heart that expresses none of the known pig glycan xenoantigens could be transplanted into a neonatal patient with no natural (preformed) anti-pig antibodies. There would be no need to develop B-cell tolerance to the three known pig antigens (as is necessary when an ABO-incompatible allograft is transplanted) because the antigens are no longer expressed. However, other protein antigens, for example, SLA, will be expressed,[6,28,29] and tolerance to these will have to be induced. This may be easier to achieve in neonates if memory cells have not yet developed. (If required, expression of SLA can be reduced by genetic engineering techniques that delete expression of SLA class I[30] or reduce expression of SLA class II.[31])

The important question, of course, is whether T-cell tolerance could be achieved. In a small number of adult patients with end-stage renal disease undergoing kidney allotransplantation, tolerance has been achieved by intensive pretransplant immunomodulation in combination with donor-specific hematopoietic progenitor cell transplantation, with the development of mixed hematopoietic cell chimerism.[32] This therapy would not be successful when pigs are the source of hematopoietic cells[33] and in any case would carry a significant risk in neonates and infants with severe cardiac insufficiency.

Based on the clinical studies of Markert and her colleagues, we propose an alternative approach that we believe would be preferable. We suggest that a combination of native thymectomy (which is almost routinely carried out in infants undergoing orthotopic heart transplantation to provide access for the surgical procedure and space for the donor heart) and donor-specific pig thymus transplantation might result in the induction of T-cell tolerance. Whether the thymectomy needs to be total or whether remnants of thymus will be detrimental (or even beneficial) to the development of tolerance is uncertain.

3 | THYMUS TISSUE TRANSPLANTATION

Postnatal allogeneic HLA-nonmatched cultured thymus tissue allotransplants in patients with complete DiGeorge (immunodeficiency) syndrome have been carried out in children under the age of 2 years (median age 5 months; range 1.1-22.1 months) since 2003.[34–36] Cultured postnatal donor-specific thymus tissue slices are transplanted at multiple sites into both quadriceps muscles of the recipient. Thymopoiesis has been observed in biopsies of the thymic tissue within 2 months, and naïve T cells have been monitored in the blood within 3-5 months.[34,35] Importantly, parental parathyroid transplantation was successful when combined with third-party allogeneic thymus transplantation.[36] Matching of the allogeneic thymus graft to the parathyroid donor HLA class II alleles that were *unshared* with the recipient appeared to be associated with the induction of tolerance toward the parathyroid graft.[36]

Neonates with complex congenital heart disease, but intact immune systems, are not comparable to patients with complete DiGeorge syndrome, as they have developed T cells during fetal life. After thymectomy (performed during the neonatal period), they become comparable to infants with incomplete DiGeorge syndrome, that is, they have some mature T cells. Nevertheless, we suggest that therapeutic depletion of existing T cells (eg, by antithymocyte globulin), followed by maintenance therapy with an anti-CD40mAb (which we have demonstrated in juvenile baboons is associated with very little recovery in the number of T cells[4]), may well induce a state of tolerance.

After depletion of T cells, the transplantation of pig thymus tissue (at the time of pig heart transplantation) may allow negative selection (and depletion) of developing antidonor T lymphocytes to enable T (and B)-cell tolerance to be achieved on a consistent basis. The pig thymus tissue would “reprogram” the recipient’s immune system to accept the pig graft as “self.”[37] The thymus graft would be from the same pig source as the heart graft, that is, it would be donor-specific. The mechanisms by which thymus transplantation might induce a state of tolerance have been explored by Sykes and her colleagues in mouse and pig models.[38–41]

Thymus transplantation has been investigated as a means of inducing tolerance to an organ allograft or xenograft in adult animals. Work in pigs and NHPs (which is of most relevance to the present approach) has largely been by Yamada, et al.[37,42,43] All of their studies have been carried out in *juvenile or adult thymectomized* pigs or baboons. Using various models of thymic tissue transplantation (eg, vascularized thymic lobes, composite thymokidneys, thymic implants into the omentum), tolerance was achieved to pig organ *allografts*, but *not* to pig organ *xenografts* in baboons, where anti-Gal antibody-mediated rejection developed despite some evidence of an effect on the T-cell response.[37]

After the transplantation into baboons of pig composite “thymokidneys” (ie, kidneys under the capsule of which autologous pig thymic tissue had been placed), Yamada’s group were able to demonstrate early baboon thymopoiesis in the porcine thymic tissue of these grafts.[44] This was associated with in vitro evidence of donor-specific T-cell unresponsiveness, but

unfortunately the baboons did not survive long enough to determine whether T-cell tolerance had been achieved.

Of relevance to the present suggested approach, Yamada's studies demonstrated that corticosteroid therapy and old age of the recipient were detrimental to the induction of tolerance. (In the proposed approach, the recipient would obviously be young and corticosteroid therapy could be avoided.) The importance of the presence of viable donor thymic epithelium and the role of the thymus during the induction phase of tolerance, particularly during the first 1-2 weeks, were emphasized.

There are, however, important differences between Yamada's studies and the present proposed approach, some of which have been mentioned above. (a) The recipients would be thymectomized neonates rather than thymectomized adults. (b) The thymic transplants would be carried out *in the absence of any antibody to pig xenoantigens* and at a time when recipient T-cell numbers and function are minimal. (c) The TKO pig donors will not express the three known carbohydrate antigens against which humans have natural antibodies and can develop elicited antibodies.[5] (d) The immunosuppressive regimen has been fully proven to inhibit both antibody-mediated and cellular rejection in several xenotransplantation models.

The additional expression of one or more human complement-regulatory proteins, for example, CD46, CD55, CD59, and one or more human coagulation-regulatory proteins, for example, thrombomodulin, endothelial protein C receptor, would enhance xenograft survival by providing increased protection against the primate immune/inflammatory response.[1] For example, a human complement-regulatory protein would protect against alternative pathway complement activation during ischemia-reperfusion injury or infection, whereas a human coagulation-regulatory protein would protect against the coagulation dysfunction seen between pigs and primates.[45,46]

4 | IMMUNOSUPPRESSIVE AND ADJUNCTIVE THERAPY

For some years, the standard regimen in the pig-to-NHP models was based on anti-CD154mAb but, because of its thrombogenic properties, alternative costimulation blockade agents have been investigated.[2,4,27] Anti-CD40mAb-based regimens have successfully prevented T cell–dependent immunity, that is, prevention of (a) an increase in the cellular proliferative response on mixed lymphocyte reaction (MLR),[47] (b) a detectable elicited antibody response, and (c) T-cell infiltration of the graft. These regimens have been combined with induction T-cell depletion with antithymocyte globulin (ATG) and B-cell depletion with an anti-CD20mAb (rituximab).[4] For maintenance therapy, the combination of costimulation blockade with either mycophenolate mofetil or rapamycin has been found effective with or without concomitant corticosteroid therapy.[48]

Using an anti-CD154mAb- or anti-CD40mAb-based regimen, features of thrombotic microangiopathy were documented in pig heart and kidney grafts in NHPs, and consumptive coagulopathy was problematic, but these complications have subsequently been eliminated by the transplantation of grafts from pigs expressing one or more human coagulation-

regulatory proteins.[2,4] It is likely that these complications develop as a result of activation of the vascular endothelium of the graft by natural (preformed) anti-pig antibody ± complement ± innate immune cells,[49] combined with molecular incompatibilities in the coagulation systems between pigs and primates. We suggest that thrombotic microangiopathy and consumptive coagulopathy are much less likely to develop early after TKO pig organ transplantation in infants (if de novo antibody production is prevented), as there should be no binding of natural anticarbohydrate antibodies to the vascular endothelium of a TKO pig heart.

As mentioned above, other genetic modifications, for example, pigs expressing CTLA4-Ig,[50] SLA Class I-KO pigs,[30] or pigs with the MHC Class II mutation (CIITA-knockdown),[31] that provide some protection from the T-cell response, could also be considered if the T-cell response proves stronger than anticipated (though reduced expression of SLA Class II might inhibit the induction of tolerance).

We have identified a substantial role for inflammation in the pig-to-NHP model, as evidenced, for example, by a sustained increase in C-reactive protein.[51] The inflammatory response may be detrimental to the induction of tolerance[52] and therefore needs to be prevented or minimized, either by drug therapy (eg, IL6-R blockade) or by further genetic engineering of the organ-source pig (eg, by expression of A20 or hemeoxygenase-1).[53] We and others have hypothesized that the inflammatory and immune responses and coagulation disturbances are mutually potentiating each other.[53]

5 | MONITORING FOR THE INDUCTION OF TOLERANCE

To determine whether donor-specific T-cell and B-cell tolerance has developed, immune monitoring would include measurement of anti-pig IgM and IgG antibodies by flow cytometry, complement-dependent serum cytotoxicity (using donor-specific pig PBMCs), T-cell and B-cell counts, and determination of T-cell and B-cell phenotypes and regulatory T cells. The ratio of regulatory T cells to memory T cells will help indicate whether tolerance has been achieved. Other assays will include functional assays of regulatory T-cell suppressor activity and CFSE-MLR (direct and indirect).

In vitro evidence of (a) an absence of anti-TKO pig antibodies (yet with, eg, the development of anti-Gal and/or anti-A/B blood group antibodies) and (b) no increase in the T-cell proliferative response to TKO pig cells on MLR (yet with an increase in the proliferative response to allogeneic cells) would strongly suggest that immunologic tolerance has been achieved. This might justify a gradual reduction in the immunosuppressive therapy, during which in vitro monitoring would continue, with eventual discontinuation.

6 | COMMENT

Cardiac allotransplantation during the first few weeks of life is associated with the best outcome of any age group and offers perhaps the best opportunity of achieving immunological tolerance to the graft. The ability to transplant a genetically engineered pig heart would overcome the substantial barrier of obtaining a heart in a timely manner.

The excellent long-term outcomes of ABO-incompatible heart allotransplantation in this age group (and of pig heart xenotransplantation in older NHP recipients) provide encouragement that, with effective immunosuppressive therapy to prevent the cellular response, a pig graft would sustain life for a clinically relevant prolonged period of time. However, even though the induction of a state of immune tolerance in the recipient should prove easier than in older children and adults, it nevertheless provides significant challenges.

The patient would need to be monitored continuously for signs of infection, autoimmunity (eg, gastrointestinal disturbances, skin rash) and a potentially immunodeficient state (suggested by the immune assays). For example, in our previous study, after receiving immunosuppressive therapy for several months, four infant baboons developed collagenous colitis.[54] However, when identified at an early stage, this condition was successfully treated with budesonide, suggesting that the baboons' immune defensive mechanisms remained intact. Nevertheless, there is a potential heightened risk of infectious (or even neoplastic) complications in infants who have not yet developed memory T cells.

The effects of many aspects of the protocol we outline are uncertain, for example, the roles that might be played by thymectomy and/or thymic transplantation, and considerable experimental data need to be accumulated before a clinical trial should be considered. Furthermore, it is possible that 12 months' immunosuppressive therapy will prove to be an insufficient period of time to enable tolerance to be achieved. On the basis of the immune assays, consideration would need to be given to prolonging immunosuppressive therapy for a longer period of time. However, even if T-cell tolerance is not achieved, prolonged survival of a genetically engineered pig heart in a recipient receiving immunosuppressive therapy would enable the heart to be employed either (a) as a short-term "bridge" to allotransplantation (probably without risk of allosensitization[55]) or (b) as long-term destination therapy. (If the initial intention of the pig heart transplant was to use it only as a "bridge," then there would be no purpose in employing the tolerance-inducing regimen, eg, thymus transplantation, as this would not, of course, induce tolerance to a subsequent allograft. Furthermore, we would not begin to reduce immunosuppressive therapy while awaiting an allograft to become available.)

We do not believe that the field has advanced sufficiently to consider cardiac xenotransplantation today, though, with the current rate of advances in the field, it will not be long before clinical trials of organ xenotransplantation will become ethically justified. We do not necessarily believe that cardiac xenotransplantation in neonates needs to be delayed until (a) cardiac xenotransplantation has been carried out in adults or (b) tolerance has been achieved to AB-incompatible cardiac allografts in neonates.

In conclusion, we believe that the development of tolerance to a genetically engineered pig heart could be achieved in immunosuppressed neonates undergoing heart transplantation within the first few days of life if combined with thymectomy and donor-specific pig thymus tissue transplantation. The likelihood of success is greater in neonates than in any other age group, and a good case can be made that clinical cardiac xenotransplantation should first be carried out in the newborn.

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Abbreviations:

mAb	monoclonal antibody
MLR	mixed lymphocyte reaction
NHP	non-human primate
PBMC	peripheral blood mononuclear cells
TKO	triple-knockout (ie, a pig that does not express the three known antigens against which humans have natural (preformed) antibodies)

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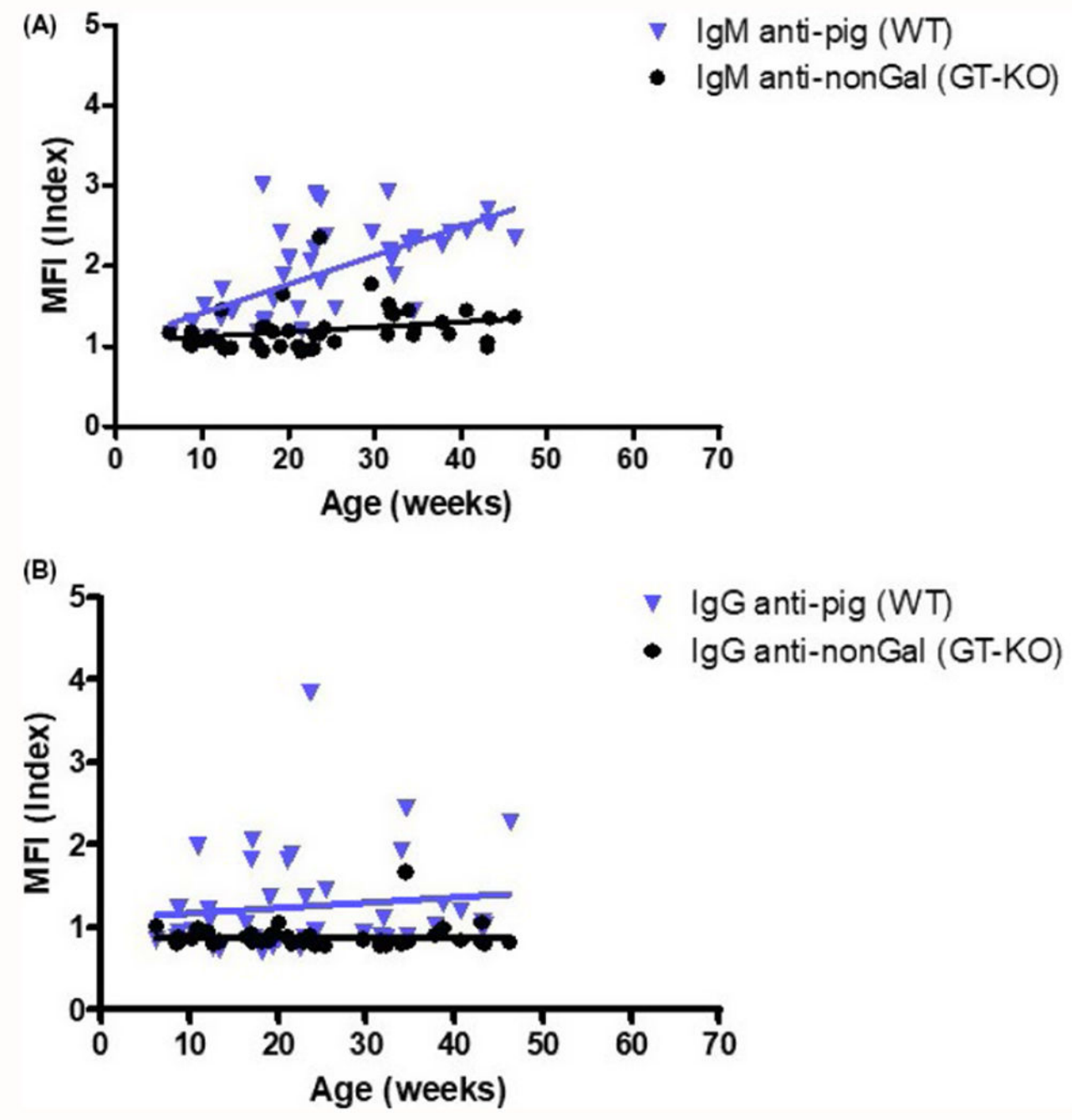

FIGURE 1.

Binding of infant human (n = 42) serum antibodies to wild-type (WT, genetically unmodified) and GT-KO pig peripheral blood mononuclear cells (PBMC) (MFI index = mean fluorescence intensity of the serum sample divided by the MFI of the isotype control sample). A, Distribution of IgM reactivity against WT and GT-KO PBMC. Correlation of MFI index with age of each group is indicated by a line (vs WT, $P = 0.073$, $r = 0.316$; vs GT-KO, $P = 0.129$, $r = 0.238$). When sera from infants were tested, only 2 of 50 serum samples showed (very low) binding of IgM to TKO pig red blood cells (not shown, Hara,

et al manuscript in preparation). B, Distribution of IgG reactivity against WT and GT-KO PBMC. Correlation of MFI index with age of each group is indicated by a line (vs WT, $P = 0.381$, $r = -0.158$; vs GT-KO, $P = 0.021$, $r = 0.356$). When sera from infants were tested, no IgG binding to TKO cells was detected in any of the 50 samples (not shown, Hara, et al manuscript in preparation). (Reproduced with permission from Rood et al Transplant Int 2007;20:1050-1058.)

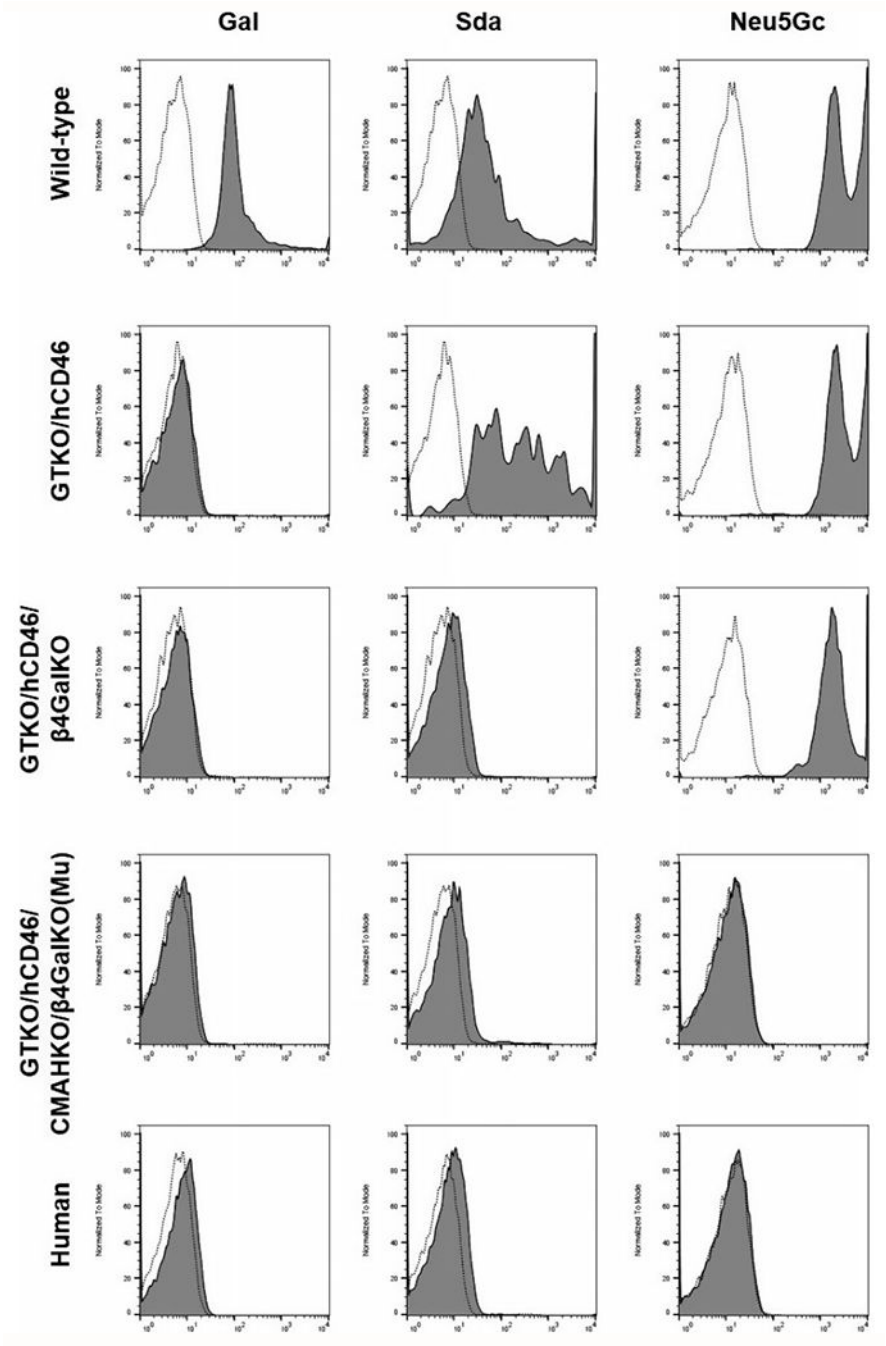

FIGURE 2.

Expression of galactose-α1,3-galactose (Gal), N-glycolylneuraminic acid (Neu5Gc), and Sda (a product of β4GalNT2-KO) on red blood cells from (top to bottom) (a) a wild-type pig, (b) a GTKO/CD46 pig, (c) a GTKO/CD46/β4GalNT2-KO pig, (d) a triple-knockout (TKO) pig (in which all three pig glycan antigens have been deleted), and (e) a healthy human. Staining was for expression of Gal (isolectin BSI-B4), Sda (dolichos biflorus agglutinin, DBA), and Neu5Gc (chicken anti-Neu5Gc mAb). RBCs from GTKO/CD46 pigs expressed Sda and Neu5Gc, but not Gal. Neither Gal nor Sda was expressed on GTKO/

CD46/β4GalNT2-KO pig RBCs. None of the 3 glycans was expressed on the TKO pig RBCs or on human RBCs