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Review

IMMUNOBIOLOGICAL BARRIERS TO PIG ORGAN XENOTRANSPLANTATION

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Summary

In suitable patients with end-stage organ failure, the transplantation of organs from living or deceased human donors offers a much-improved quality and length of life. However, the availability of deceased human donor organs is grossly inadequate. Gene-edited pigs might provide an alternative source of organs for clinical transplantation (xenotransplantation). However, there are major immunobiological barriers to successful pig organ transplantation in human or nonhuman primate recipients. These barriers include antibody binding, activation of complement, the innate cellular response, coagulation dysregulation between pig and primate, and a systemic inflammatory response, in addition to the T cell response. These have steadily been overcome by a combination of (i) genetic engineering of the organ-source pig (aimed mainly at the innate immune response), and (ii) the administration of novel immunosuppressive agents (directed towards the adaptive immune response). The immunological barriers that remain relate to both the innate and adaptive immune responses. Pig kidney transplants have now supported immunosuppressed (anephric) nonhuman primates for periods in excess of a year and pig heart transplants for up to 9 months, although these encouraging results cannot yet be achieved consistently.

Key words: gene-editing, immunosuppressive therapy, organ, pig, xenotransplantation

Abbreviations

AMR: antibody-mediated rejection
GTKO: 3-galactosyltransferase gene-knockout
HLA: human leukocyte antigen
NHP: nonhuman primate
PERV: porcine endogenous retrovirus
SLA: swine leukocyte antigen
TKO: triple-knockout

INTRODUCTION

The shortage of organs from deceased human donors for transplantation into patients with end-stage organ failure is a worldwide problem. The most likely alternative source of organs is xenotransplantation (cross-species

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transplantation), specifically the transplantation of gene-edited pig organs into human recipients. Although patients with terminal heart failure can receive a mechanical support or replacement device, kidney failure, with the exception of dialysis, can only be treated successfully by transplantation. Given the complexity of the numerous cellular functions of the kidney, bioengineering of new kidneys will be difficult and unlikely to provide a solution within the foreseeable future [1,2]. Xenotransplantation is therefore the hope for the near future.

From an immunologic perspective, nonhuman primates (NHPs) would be the preferred sources of organs for transplantation into humans, but virtually all of these species are either endangered or are too small to provide organs suitable for transplantation into large adult humans. Furthermore, concerns have been raised about the transmission of infectious agents from NHPs to humans, particularly since most NHPs are either wild-caught or have been housed under colony conditions for relatively few generations. The time and expense of breeding these animals in captivity are also prohibitive, as is a lack of experience in genetically modifying them. In addition, many members of the public would object to the use of NHPs on ethical grounds.

The pig has therefore been identified as the species most likely to be the source of organs for clinical xenotransplantation, and in recent years research efforts have been directed toward pig-to-NHP transplantation. There are several advantages for using the pig as an organ-source [3]. However, a major disadvantage is that the human and NHP immune response to organs from wild-type (i.e., genetically-unmodified) pigs is rapid and intense, resulting in hyperacute rejection.

If the pig could be the organ-source, there are several potential advantages of xenotransplantation when compared to allotransplantation. The availability of an unlimited number of organs whenever required is just the most obvious. Others include the potential for infection-free organs that have not been damaged by the adverse effects of brain death or cessation of heartbeat. Xenotransplantation provides us with the first real opportunity (in > 70 years of clinical transplantation) of modifying the donor, rather than just treating the recipient. The more we can do to the donor, the less we will need to do to the recipient [4-7]. This should eventually result in the need for minimal or no immunosuppressive drug therapy, leading to fewer adverse events.

IMMUNOBIOLOGICAL BARRIERS TO PIG ORGAN XENOTRANSPLANTATION

All humans and Old World NHPs have 'natural' antibodies to pig xenoantigens, which they develop during the first year of life (Fig. 1) as a defensive mechanism when their gastrointestinal tract is colonized by potentially pathogenic microorganisms that express some of the same antigens as pigs [8-10].

Antibody-mediated rejection (AMR) is therefore common after pig organ transplantation into Old World NHPs, even when the organ is taken from a triple-knockout (TKO) pig, i.e., a pig in which the expression of the three known pig glycan xenoantigens has been deleted (Tab. I). Whether AMR is related to the presence of natural (preformed) anti-glycan antibodies in these NHPs or to the development of elicited antibodies directed to other glycan or protein antigens expressed on the pig cells, e.g., swine leukocyte antigens (SLA), remains uncertain, but is probably associated with both. As in allotransplantation, AMR can be acute or chronic.

In our experience in xenotransplantation we have never successfully reversed acute AMR and, to our knowledge, nor has any other research group. As all NHPs have pre-existing antibodies to TKO pig organ grafts, i.e., they are sensitized [11-14], most research groups now select NHPs will low anti-pig antibody levels for their pig organ transplantation experiments. However, there is still a risk of early AMR from natural antibody or from elicited antibody (if the immunosuppressive therapy is inadequate).

Many humans, however, do not have antibodies to TKO pig xenoantigens, and in vitro studies suggest that early AMR will not occur when TKO pig organs are transplanted clinically (if the adaptive immune response is suppressed successfully) [15].

THE MECHANISM OF AMR IN XENOTRANSPLANTATION

Xenoreactive natural antibodies

Circulating natural (or preformed) antibodies are immunoglobulins found in the serum of healthy humans and NHP species without known antigenic stimulation. As part of the innate immune response, they play a key role in the recognition and neutralization of pathogens and in the stimulation of phagocytic macrophage activity. In the context of pig-to-primate organ xenotransplantation, natural antibodies to glycan xenoantigens expressed on the pig cells (Tab. I) initiate rejection through activation of the classical complement pathway [16,17].

Several studies have demonstrated that antibodies of IgM isotype are the main immunoglobulins involved in the onset of acute AMR [18], though IgG, IgA, and IgE antibodies are present[19]. Anti-pig aortic endothelial cell IgM antibodies are more efficient in activating the classical pathway of complement than anti-pig IgG natural antibodies [20,21]. Within the pool of IgG xenoreactive antibodies, IgG1 and

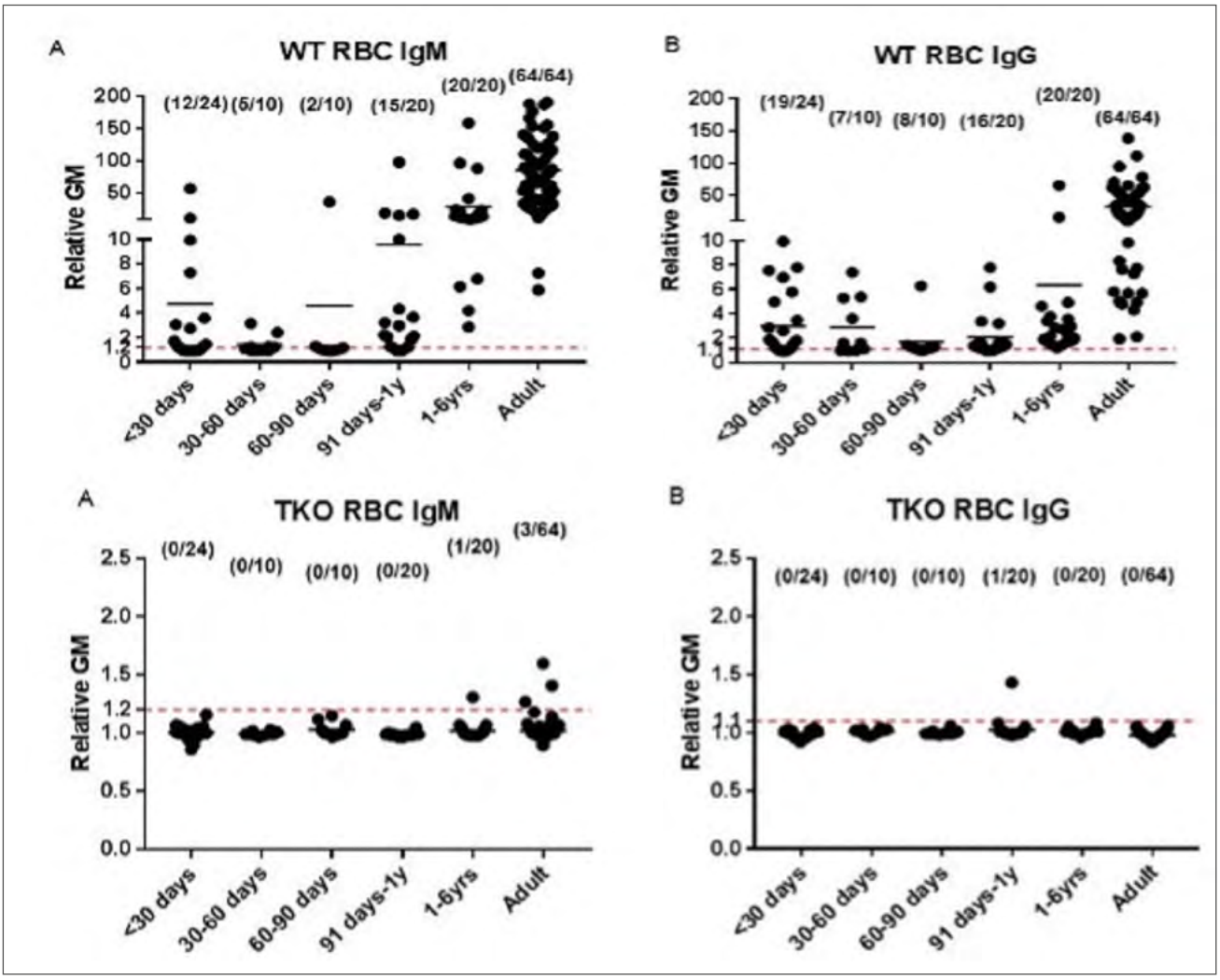


Figure 1. Human serum antibody binding to pig red blood cells by age. **(Top):** geometric mean (GM) binding and age correlation of human serum IgM (A) and IgG (B) antibodies to wild-type (WT) pig red blood cells (RBCs). There is a steady increase in IgM and IgG binding during the first year of life. **(Bottom):** geometric mean (GM) binding and age correlation of human serum IgM (A) and IgG (B) antibodies to TKO pig RBCs. There is virtually no increase in IgM or IgG antibodies during the first year of life, and a very low level of antibodies in adults. The dotted lines indicate no IgM or IgG binding. (Note the great difference in the scale on the Y axis between Top *vs* Bottom (reproduced with permission from Li Q et al. Ann Thorac Surg 2020;109:1268-1273).

Table I. Known carbohydrate xenoantigens expressed on pig cells.

Carbohydrate (Abbreviation)	Responsible enzyme	Gene-knockout pig
1.Galactose-α1,3-galactose (Gal).	α1,3-galactosyltransferase	GTKO
2.N-glycolylneuraminic acid (Neu5Gc).	CMAH	CMAH-KO
3.Sda	β-1,4N-acetylgalactosaminyltransferase.	β4GalNT2-KO

CMAH = Cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH).

IgG2 subclasses are most abundant. While IgG1 and IgG3 can activate the classical pathway of complement, lgG2 can only activate the alternative complement pathway [22,23]. However, in comparison to IgM, a much higher concentration of IgG antibodies is needed to achieve complement activation.

Xenoreactive natural antibodies can be directed to Gal (anti-Gal) or to nonGal (anti-nonGal) antigens. Anti-Gal antibodies account for approximately 1% of circulating immunoglobulins [24-28]. Deletion of expression of Gal in the organ-source pig largely prevents hyperacute rejection [29,30], but, in the absence of effective immunosuppressive therapy, does not prevent acute AMR [31,32].

A more recent evolutionary loss of cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH), an enzyme involved in sialic acid synthesis, led to the unique absence of the glycolyl form of neuraminic acid (Neu5Gc) in humans (Tab. I). All other mammals (except New World NHPs) express both the acetyl form of neuraminic acid (Neu5Ac) and the glycolyl form (Neu5Gc) at various ratios in their glycoproteins and glycolipids. In some humans, exposure to Neu5Gc expressed in food (especially milk and red meat) can induce production of anti-Neu5Gc IgG and IgM antibodies [33-36]. Antibodies to Neu5Gc appear to play a greater role in the Chinese than in Western populations [37]. Because all Old World NHPs express Neu5Gc, the pig-to-Old World NHP model is not suitable for investigating the effect of anti-Neu5Gc antibodies on pig xenografts [12,13,38-41].

With regard to the third known pig glycan xenoantigen, Sda (Tab. I), although commonly expressed on human gastrointestinal epithelial cells and some other tissues and on human red blood cells (RBCs), most humans produce low levels of cold-reactive anti-Sda IgM, making Sda a polyagglutinable RBC antigen. While the Sda blood group does not carry a significant transfusion risk, Sda expression on pig vascular endothelial cells may induce an antibody response in a primate host [42-44]. Antibodies to Sda appear to play a greater role in the pig-to-NHP model than they will in clinical xenotransplantation [45].

The genes for the three key enzymes responsible for the production of xenoglycans in the pig have successfully been knocked out (Tab. I), producing TKO pigs. While many humans exhibit no or minimal antibody binding to cells from TKO pigs (Fig. 1) [46], complement activation and coagulation pathway dysregulation may still be observed, in part associated with molecular incompatibilities between the species [47,48]. This particularly pertains to the inefficient binding of human complement and coagulation pathway proteins to pig complement-regulatory and thromboregulatory molecules, respectively.

Although clearly beneficial when a pig organ is to be transplanted into a human recipient, there are problems with TKO pig organ transplantation in Old World NHPs (Fig. 2). As all Old World NHPs express the Neu5Gc antigen, these species do not develop anti-Neu5Gc antibodies (Tab. I). Estrada et al [39] reported that, when the Neu5Gc antigen is deleted in pigs, it appears to expose another xenoantigen against which Old World NHPs, but not humans, have natural antibodies. IgM and IgG binding are higher to TKO pig cells than to GTKO cells, and serum cytotoxicity is greater than to pig cells in which Neu5Gc remains expressed (Fig. 3).

However, there are other possible contributing factors, e.g., relating to complement, that may be playing a role in the high level of NHP serum cytotoxicity to TKO pig cells (Fig. 4) [12,49]. The observation that approximately half of the baboon sera tested demonstrate a high level of cytotoxicity to double-knockout pig cells (i.e., those that do not express Gal or Sda but continue to express Neu5Gc) (Fig. 3) suggests that other factors (than absence of Neu5Gc expression) are involved. Although hyperacute rejection is rare, TKO pig-to-NHP organ transplantation still results in a relatively high incidence of early graft failure from AMR (Fig. 5) [38,50], although therapy with an anti-CD154 agent appears to overcome this barrier in some cases (see below) [51]. Because all Old World NHPs have cytotoxic antibodies to TKO pig cells, the pig-to-NHP model is no longer representative of clinical pig organ xenotransplantation and has led some to advocate for the initiation of limited exploratory clinical trials [13,52].

Complement activation

The complement system is a collection of circulating and cell membrane proteins that play important roles in host defense against non-self antigens, including microbes and, unfortunately, organ grafts [17] (Fig. 6). It can be activated by these non-self antigens in the absence of antibody, as part of the innate immune response (alternative and lectin pathways) or when antibodies attach to non-self antigens (classical pathway). The complement system is important in the development of ischemia-reperfusion injury and delayed graft function, as well as in both acute and chronic AMR.

This role of complement in the humoral immune response illustrates the fundamental tenet of the two-signal hypothesis, namely that in addition to recognition of the antigen, the innate immune response to these antigens provides additional signals that are necessary for lymphocyte activation. Complement proteins bound to antigen-antibody complexes are recognized by follicular dendritic cells in germinal centers, allowing the antigen to be displayed for further B cell activation and selection of high-affinity B cells. This process is an example of an innate immune response to a non-self antigen (complement activation) enhancing an adaptive immune response to the same antigen (B cell activation and antibody production).

The binding of human serum antibodies to the xenoantigens expressed on wild-type (i.e., genetically-unmodified) pig organs results in almost immediate activation of the complement cascade, and the graft is destroyed (hyperacute rejection) [53,54]. This very rarely occurs after the transplantation of TKO pig organs, but it can occur if preservation of the graft has been inadequate (Cooper DKC et al., unpublished observations) (as ischemia can

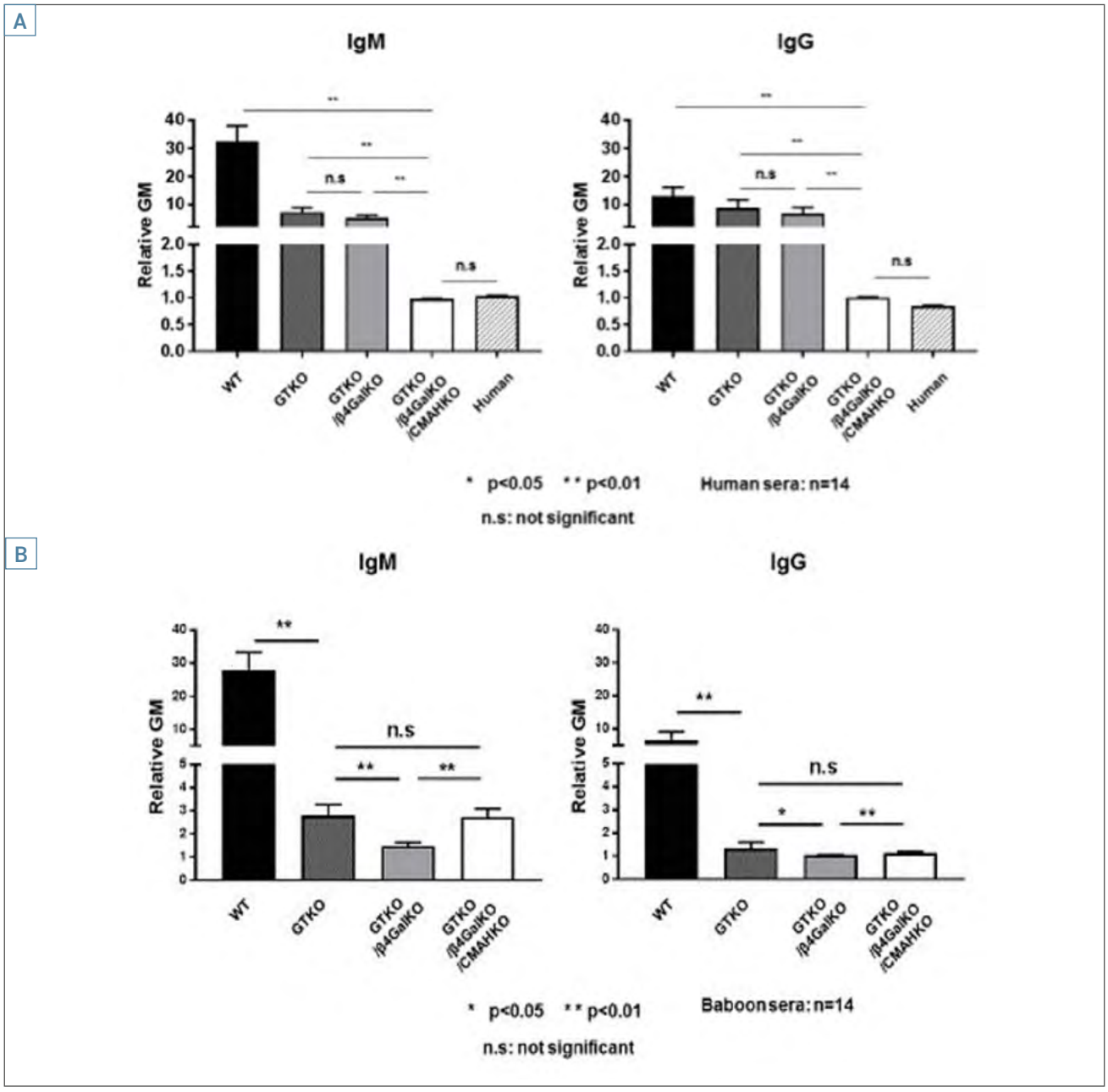


Figure 2. A) Human (top) and baboon (bottom) serum antibody binding to red blood cells (RBCs) from various pigs. Human serum antibody binding to pRBCs was measured by flow cytometry using the relative geometric mean (rGM), which was calculated by dividing the GM value for each sample by the negative control. Negative controls were obtained by incubating the cells with secondary anti-human antibodies only (with no serum). (Top) Human serum (n = 14) IgM (left) and IgG (right) antibody binding to wild-type (WT), GTKO, double-knockout (i.e., deletion of expression of Gal and Sd[a]), and triple-knockout (TKO, i.e., with additional deletion of expression of Neu5Gc) pRBCs. Human IgM and IgG binding to GTKO/4GalKO/CMAHKO (TKO) pig RBCs was almost at the level of binding to human RBCs, and there was no detectable IgM or IgG binding to TKO RBCs. Binding to TKO pig RBCs was not significantly different from human IgM and IgG binding to human RBCs of blood type O. (*p < 0.05; **p < 0.01; ns = not significant); **B)** Baboon (an Old World NHP, n = 14) IgM and IgG antibody binding to WT, GTKO, DKO, and TKO pig RBCs. (Note that deletion of Neu5Gc [CMAH-KO] in pig cells appears to expose a fourth xenoantigen against which baboons have natural IgM antibodies. Note also that the data support the observation that the deletion of expression of Gal has more effect in reducing the antigenicity of baboon serum (90% reduction) (**A**), when compared with human serum (70% reduction) (*p < 0.05, **p < 0.01; ns = not significant) (reproduced in part with permission from Cooper DKC et al. Xenotransplantation 2019;26:E12516. https://doi.org/10.1111/xen.12516).

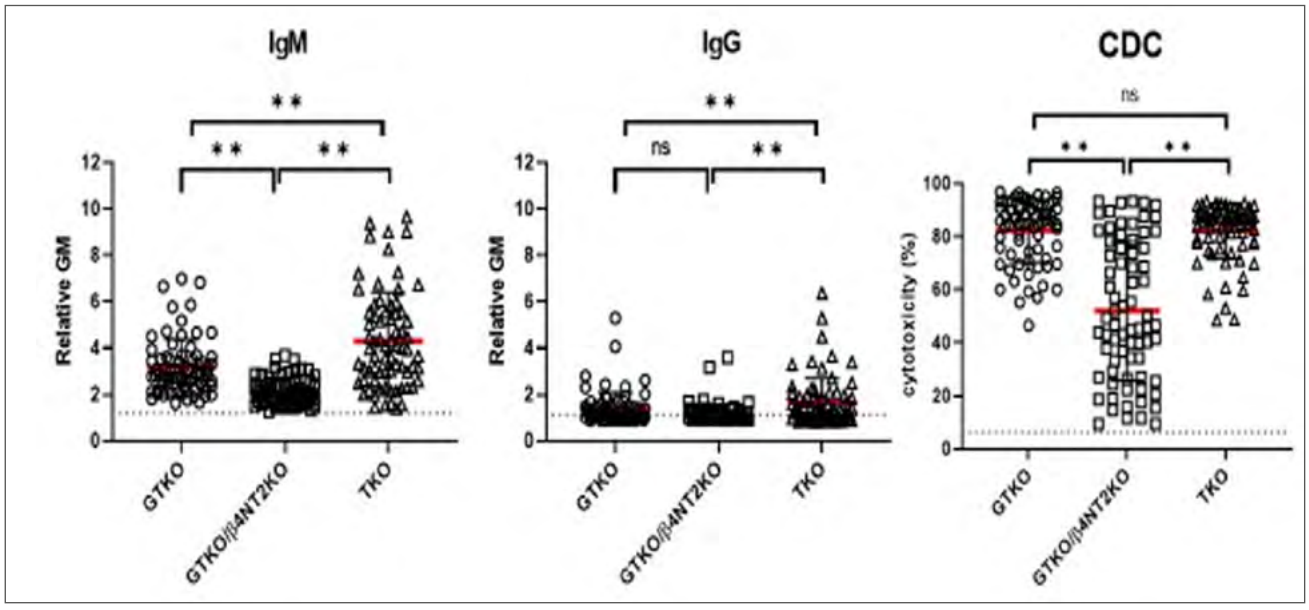


Figure 3. Comparison of mean serum IgM (left)/IgG (**middle**) binding and serum complement-mediated cytotoxicity (CDC, **right**) of baboons (n = 72) to GTKO, GTKO/4GalNT2KO, and TKO pig peripheral blood mononuclear cells (PBMCs). On the y axis, the dotted line represents the cut-off values (IgM [rGM] 1.2, IgG [rGM] 1.1, CDC 6.4%) below which there is no binding or cytotoxicity. The red lines indicate the mean values. (*p < 0.05; **p < 0.01; ns = not significant). IgM and IgG binding and serum cytotoxicity to TKO cells were higher or comparable to binding to GTKO cells. Although mean IgM and IgG binding and mean serum cytotoxicity to DKO cells were less than to TKO cells, many baboons had a high level of cytotoxicity to DKO cells (**p < 0.01) (reproduced with permission from Yamamoto T et al. Sci Rep 2020;10:9771. https://doi.org/10.1038/s41598-020-66311-3).

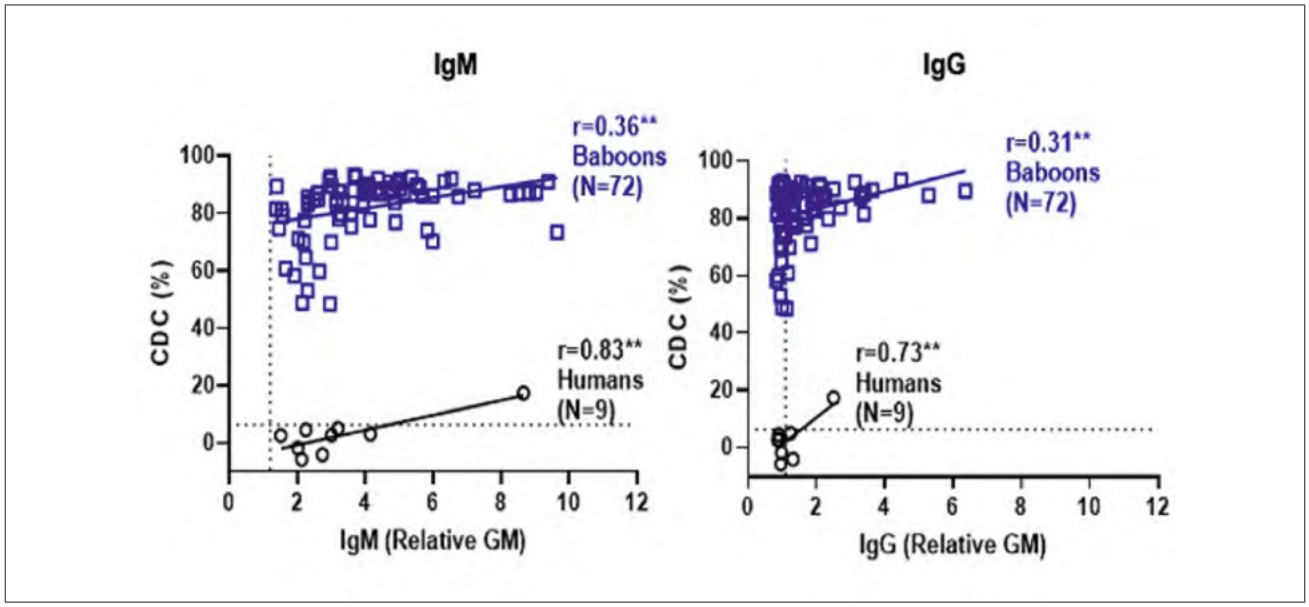


Figure 4. Correlation of human (n = 9) and baboon (n = 72) serum IgM (**left**) and IgG (**right**) antibody binding with serum complement-dependent cytotoxicity (CDC, at 50% serum concentration) to TKO pPBMCs. In both humans and baboons, there was a significant increase in cytotoxicity as IgM and IgG antibody binding to TKO pPBMCs increased. In baboons, however, cytotoxicity was high whether IgM binding was high (e.g., 80% cytotoxicity at a rGM of 8), or relatively lower (e.g., 75% at a rGM of 2) (**p < 0.01) (reprinted with permission from Yamamoto T et al. Xenotransplantation 2020;27:E12596. https://doi.org/10.1111/xen.12596).

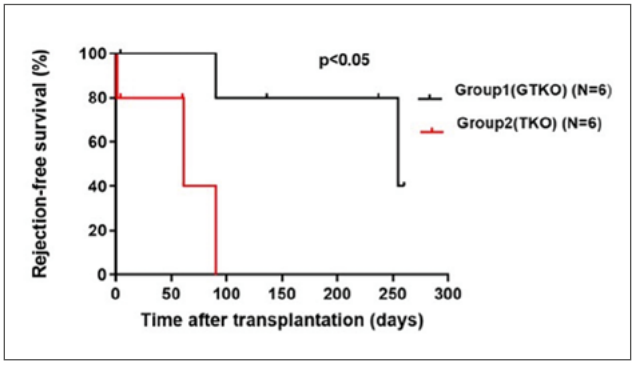
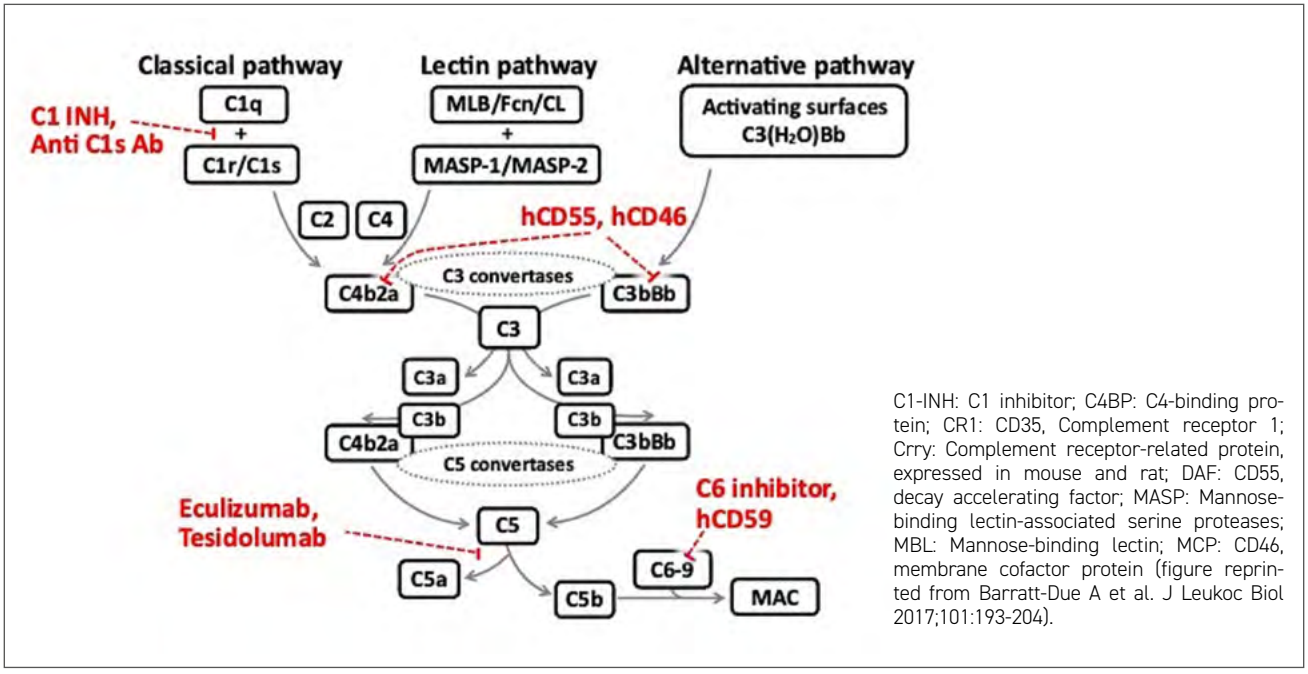


Figure 5. Rejection-free survival of GTKO pig kidneys in baboons (Group 1, **in black**) was significantly longer than that of TKO pig kidneys (Group 2, **in red**) (reproduced with permission from Iwase H, et al. Xenotransplantation 2021;28:E1700. https://doi.org/10.1111/xen.12700).

result in activation of the vascular endothelial cells). Furthermore, steps have been taken to protect the pig organ from the deleterious effects of complement activation, either by drug therapy, e.g., a C1-esterase inhibitor [55] or a C5 inhibitor, or by modifying the donor pig to express one or more human complement-regulatory proteins (e.g., CD46, CD55, CD59). Pig complement-regulatory proteins expressed on pig vascular endothelial cells are effective at protecting pig cells from the effects of pig complement, but are not successful in protecting against human complement-mediated injury[16,56-58].

White's group and others demonstrated significantly prolonged survival of pig kidneys and hearts in NHPs treated with cyclosporine-based immunosuppressive therapy when human CD55 (decay-accelerating factor) was expressed on the pig vascular endothelial cells [59].



C1-INH: C1 inhibitor; C4BP: C4-binding protein; CR1: CD35, Complement receptor 1; Crry: Complement receptor-related protein, expressed in mouse and rat; DAF: CD55, decay accelerating factor; MASP: Mannose-binding lectin-associated serine proteases; MBL: Mannose-binding lectin; MCP: CD46, membrane cofactor protein (figure reprinted from Barratt-Due A et al. J Leukoc Biol 2017;101:193-204).

Figure 6. Schema of complement system. **Classical pathway (left):** activated by binding of antibodies to antigens, which triggers C1q, activates C1r, C1s, then cleaves C4 and C2 to form C4b2a (C3 convertase); **Lectin pathway (middle):** one of MBL, ficolin -1, -2 and -3, and collectin 10/11 and collectin-P, recognizes lipopolysaccharides, etc., and binds to one of the MASP-1, MASP-2, and MASP-3, forming C3 convertase (C4b2a) (middle). C4b2a from the classical or lectin pathway cleaves C3 into C3a and C3b. C3b binds to C4b2a to form one of the C5 convertases (C4b2a3b); **Alternative pathway (right):** C3 undergoes spontaneous hydrolysis to form C3(H2O), which binds to factor B, forming an unstable C3 convertase C3(H2O)Bb, generating more C3b. Activation of C3 in the presence of factor B and factor D results in the formation of C3bBb (C3 convertase) (right). Properdin stabilizes C3 and C5 convertase, and enhances the amplification loop of C3 activation, then generating C5 convertases (C3bBb3b); **Activation of MAC (bottom)**: the C5 convertase cleaves C5 into C5a and C5b, the latter interacting with C6–C9 to form the MAC (C5b-9), which in turn results in lysis, damage, or activation of target cells (lower part). The complement system is tightly regulated by soluble inhibitors (yellow), including C1-INH, factor H (FH), factor I (FI), C4BP, anaphylatoxin inhibitor (AI) inactivating the anaphylatoxins (e.g., C5a to C5a-desArg), vitronectin (VN, S-protein, Vn, and Clusterin (CL, apolipoprotein J, SP-40) maintaining continuous low-grade activation in the fluid phase in check. Host cell membranes are equipped with a number of inhibitors to protect them against attack by complement (right), including CD46, CR1, CD55, thus controlling C4 and C3 activation. CD59 protects against final assembly of the C5b-9 complex.

The combination of GTKO and one or more human complement-regulatory proteins further prolongs graft survival [60,61].

The innate cellular response

In addition to complement activation, xenoreactive natural antibodies can lyse target cells by complement-independent pathways (i.e., by antibody-dependent cellular cytotoxicity [ADCC]). The Fab portion of xenoantibodies can bind to donor endothelial cells and the Fc receptors of innate immune cells. This triggers innate immune responses that lead to endothelial cell lysis, cytokine release, and amplification of the T cell response. Innate immune cells, e.g., macrophages, monocytes, and natural killer (NK) cells, also play significant roles [62], though these are less well-defined. The innate immune response can be inhibited by certain genetic modifications in the donor pig, e.g., expression of human CD47 and/or HLA molecules (HLA-E and/or G) that suppress macrophage and NK cell activation, respectively [63-67].

CD47's most critical function is as a marker of self-recognition. The binding of CD47 to its ligand, signaling regulatory protein (SIRP)-, inhibits macrophage function and prevents phagocytosis of cells and platelets [66,68-70]. CD47/SIRP-incompatibility, as in xenotransplantation, may also induce innate immune cell activation [71]. To overcome this incompatibility, human CD47 has been transgenically expressed in the organ-source pig [72].

Coagulation dysfunction

Several early research groups provided evidence to indicate that there were several incompatibilities between the coagulation systems of pigs and primates [47,73]. This was first clearly demonstrated in the pig-to-NHP model by Ierino et al. [74] and Kozlowski et al. [75]. As a result of the accumulation of platelets and fibrin in the pig graft, a thrombotic microangiopathy developed, impairing the function of the graft, and leading to fatal consumptive coagulopathy [76,77].

Steps were taken to express at least one human coagulation-regulatory protein, e.g., thrombomodulin (TBM), endothelial protein C receptor [EPCR], tissue factor pathway inhibitor (TFPI), and/or CD39, on the pig vascular endothelial cells. Survival of pig kidneys and hearts transplanted into NHPs was extended to months rather than weeks [78].

Systemic inflammation

The importance of the inflammatory response to both allografts and xenografts is becoming ever more widely recognized (Fig. 7) [79]. Factors that must be considered include the presence of inflammation in the recipient at the time of the transplant, e.g., associated with pre-existing comorbidities and/or chronic dialysis, and in the donor, e.g., as a result of brain death or cardiac arrest. Further inflammatory events may result from the surgical procedures. Inflammation activates recipient immune cells, e.g., neutrophils, monocytes, macrophages (which in turn produce more proinflammatory cytokines), and inflammation-mediated donor endothelial cells.

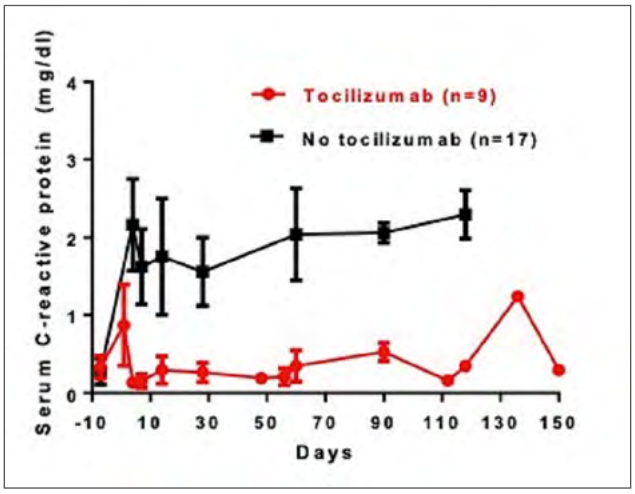


Figure 7. Serum C-reactive protein (C-RP) responses to gene-edited pig kidney or artery patch transplants in immunosuppressed baboons being treated with or without tocilizumab (anti-IL-6RmAb) (reproduced with permission from Li T et al. Transplantation 2017;101:2330-2339).

Despite the above gene-edits, evidence accumulated that suggested that, after pig organ transplantation in NHPs, systemic inflammation developed before coagulation dysfunction was obvious [80]. The insertion of a human 'apoptotic or 'anti-inflammatory' gene, e.g., hemeoxygenase-1 or A20, proved beneficial [72].

However, systemic anti-inflammatory drug prophylaxis in the form of an anti-interleukin-6 (IL-6) receptor mAb may also be advantageous. The anti-IL-6 receptor-blocking mAbs that have been tested, e.g., tocilizumab, block the binding of IL-6 to baboon tissue receptors, but not to pig tissues, and so their beneficial effect on the transplanted pig organ is questionable, and may even be detrimental [81]. Similarly, agents that bind to soluble IL-6, e.g., siltuximab, were also found to bind only to baboon IL-6 but not to pig IL-6 [81]. However, the overall effect of IL-6 blockade is generally believed to be beneficial [78].

HISTOPATHOLOGICAL FEATURES OF ACUTE AMR

Immune injury after organ xenotransplantation results in an activated endothelium which leads to apoptosis and necrosis of individual endothelial cells [82]. Acute AMR is characterized by endothelial injury, typically in the form of

microvascular inflammation, thromboses, endothelialitis, and/or transmural vasculitis, often associated with evidence of antibody and/or complement deposition (Fig. 8). Glomerular and peritubular capillary mononuclear cells are typically present and characterize the microvascular inflammation. Subendothelial cellular infiltration or endothelial loss or detachment of the larger arteries may also be seen.

In heart xenografts, the pathologic features include interstitial edema, hemorrhage, thromboses, and myocyte necrosis [83,84], initially observed as venous thromboses, associated with capillary endothelial activation and congestion, which later can be seen as regions of interstitial hemorrhage. Myocyte coagulative necrosis can be present at a later phase [85].

THE ADAPTIVE (T AND B CELL) IMMUNE RESPONSE

When hyperacute rejection was prevented by judicious gene-editing, attention turned to the suppression of the adaptive immune response, particularly to the suppression of the T cell response. T cell-dependent elicited antibody production may be playing a major role in the development of AMR, e.g., after primate exposure to swine leukocyte antigens (SLA), which are immunogenic across species [86-89]. T cell activation leads to the destruction of the graft, either by the T cells themselves or by stimulation of B cells, resulting in AMR. To overcome this barrier, immunosuppressive therapy is administered (as in allotransplantation).

Gollackner demonstrated that elicited antibodies induce endothelial cell activation and tissue factor expression far more strongly than natural antibodies and without the need for complement activation [90]. Inadequate immunosuppressive therapy resulted in early AMR even when GT-KO pig organs were transplanted [32]. Although prevention of the initial T cell response would seem to be essential, once AMR has developed the depletion of existing T cells, e.g., by ATG, would seem unlikely to reverse the process.

In 2000, Buhler and his colleagues demonstrated that conventional (cyclosporine-based) immunosuppressive therapy did not prevent rejection to pig xenoantigens from occurring (Fig. 9) [91,92]. However, this could be prevented (or at least delayed) by administration of an anti-CD154mAb to the NHP recipient. Since then, blockade of the CD40/CD154 costimulation pathway has formed the basis of all successful immunosuppressive regimens in xenotransplantation until the present day [91,93,94].

The anti-CD154mAbs available in 2000 were soon found to be thrombogenic [95-97], resulting in their withdrawal for several years until the recent introduction of Fc-modified anti-CD154 agents that are not thrombogenic. During the interim, anti-CD40mAbs, which are not thrombogenic, were administered and resulted in greatly prolonged survival of heterotopically-placed hearts (in the abdomen) in the pig-to-baboon model [98,99]. (A humanized version of this agent formed the basis of the regimen used in the clinical pig heart transplant carried out at the University of Maryland at Baltimore in 2022 [100].) There is increasing evidence, however, that in xenotransplantation anti-CD154 agents are preferable to anti-CD40 agents [101-103].

Although blockade of the CD40/CD154 costimulation pathway was successful, blockade of the B7/CD28 pathway, e.g., by CTLA4-Ig, was less so. Nevertheless, genetic engineering enabled CTLA4-Ig to be produced by the organ-source pig [104]. The production of CTLA4-Ig was

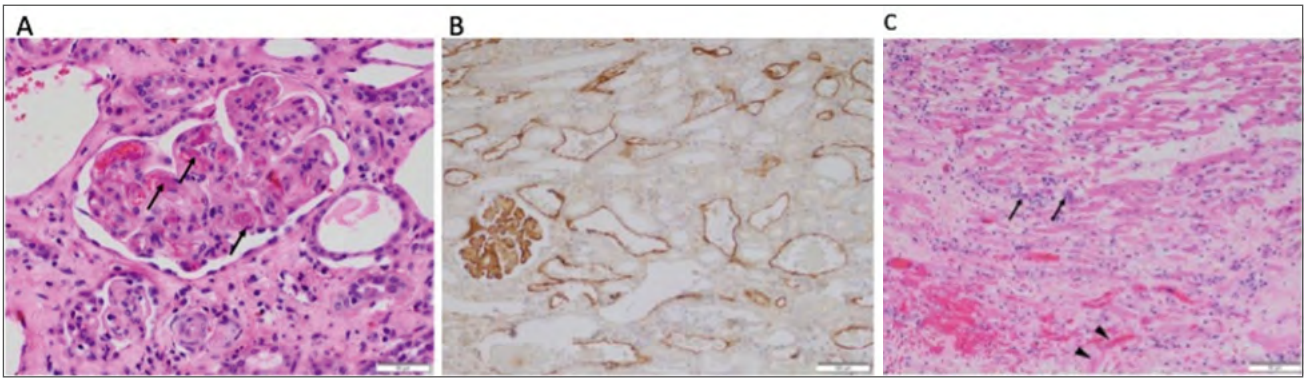


Figure 8. Histopathology of AMR in pig kidney and heart grafts transplanted into immunosuppressed NHPs. **A)** AMR in a pig kidney xenograft showing glomerular intracapillary thrombi (black arrows). Other capillaries of the glomerulus show congestion, fibrin and segmental endothelial swelling and cell loss (H&E, 400x); **B)** C4d deposition is present along peritubular and glomerular capillaries (C4d immunoperoxidase, 200x); **C).** AMR in a pig heart xenograft showing extensive interstitial edema, intracapillary mononuclear cells (arrows) and capillary thrombi (arrowheads). Interstitial hemorrhage is also evident (H&E, 400x).

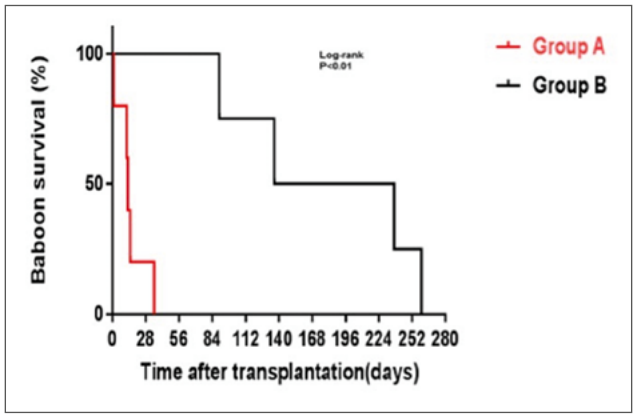


Figure 9. GTKO pig kidney graft survival in baboons receiving conventional (tacrolimus-based) US FDA-approved immunosuppressive agents (Group A, **in red**) was much shorter than in those receiving an anti-CD40mAb-based regimen (Group B, **in black**) (reproduced with permission from Yamamoto T et al. Transplantation 2019;103:2090-2104).

so extensive that the pigs became immunosuppressed, resulting in a high incidence of infectious complications, limiting survival. The approach of expressing an immunosuppressive agent only in the specific cells of interest (e.g., pancreatic islets) has been further explored [105] and has potential for the future.

Costimulation blockade is currently combined with a conventional agent, e.g., rapamycin or mycophenolate mofetil (MMF) [106,107] (Tab. II).

Based on (i) the innovative biotechnology for pig gene modification aimed at reducing the effect of the primate immune response to the xenograft, and (ii) the administration of novel immunosuppressive agents that block the CD40/CD154 costimulation pathway, significant progress has been made in pig-to-NHP organ xenotransplantation models [51,78,108-110]. These advances have led to prolonged survival of pig kidney grafts in NHPs, and today survival is being recorded in months or years.

Potential remaining immunological challenge: control of indirect T cell recognition

In allotransplantation, the production of donor-specific HLA antibodies (DSAs), resulting from interaction between antigen-presenting cells (APCs, including B cells) and T cells through the indirect recognition pathway, hinders long-term graft survival. DSA production depends not only on the amino acid differences in the B cell epitopes recognized by the B cell receptors, but also on the type and number of T cell epitopes recognized by the T cell receptors, i.e., the donor protein (mismatched HLA)-derived unique core peptides presented by recipient HLA class II [111].

In xenotransplantation, the number of epitopes is presumably much higher. Antibody production requires strong T cell help because more peptides can be presented on xenografts than on allografts [112]. Many studies have been conducted on B cell epitopes, and Gal, Neu5Gc, and Sda have attracted attention as natural anti-pig antibodies. T cell epitopes have been studied with a focus on direct recognition pathways, e.g., by the mixed lymphocyte reaction (MLR, i.e., reactions between donor antigen-presenting cells with recipient T cells).

In contrast, assays for the indirect pathway (reactions between donor-derived peptides presented by recipient APCs to recipient T cells) in xenotransplantation have not yet been fully developed and therefore have not been adequately studied [113]. Naïve NHPs and humans have usually not been exposed to pig antigens, i.e., they are

Table II. Representative immunosuppressive regimen.

Agent	Dose (duration)
Induction	
Thymoglobulin (ATG)	5 mg/kg i.v. (day -3) (to reduce the $CD3^+$T cell count to <500/mm^3)
Anti-CD20mAb (rituximab)	10 mg/kg i.v. (day -2)
C1-esterase inhibitor	17.5 U/kg i.v. (days 0, 2)
Maintenance	
Anti-CD154 monoclonal antibody (mAb)	Dose dependent on the agent used (days 0, 2, 7, 10, 14, and weekly)
Rapamycin	mg/kg i.m. daily (target trough 6-10 ng/ml), beginning on day -7.
Methylprednisolone	10 mg/kg/d on day 0, tapering to 0.25 mg/kg/d by day 7.
Anti-inflammatory	
Tocilizumab	8 mg/kg i.m. on days 0, 7, 14, and then monthly
Adjunctive	
Aspirin	40 mg p.o. (alternate days), from day 4.

not sensitized. Therefore, their immune response to pig antigens might not be detected by an indirect MLR. *In vivo* studies, e.g., organ transplantation, will be necessary to assess the T cell response through the indirect pathway by indirect MLR [113].

Theoretically, complete blockade of the CD40/CD154 pathway should control T cell help and suppress the immune response to the donor, but it would also suppress the immune response to infection and vaccines, and so intensive immunosuppressive therapy may not be desirable.

CROSS-REACTIVITY OF ANTIBODIES BETWEEN HLA AND SLA

Although many patients with antibodies to HLA do not appear to be at any increased risk of rejection of a pig organ graft [14,44,46], cross-reactivity between anti-HLA antibodies and swine leukocyte antigens (SLA) may occur, although the incidence is low (< 5%) [14]. If patients with anti-HLA antibodies that do not cross-react with SLA are identified by in vitro assays [114], then these patients should be acceptable for the initial clinical trials. For the future, methods are being developed to delete or replace specific SLA against which there might be cross-reactivity [87,115].

Of importance, if a patient receives a pig organ that is rejected with the development of elicited anti-pig antibodies, e.g., against SLA. the current (limited) evidence is that this will not preclude subsequent successful allotransplantation [14]. In clinical trials, therefore, pig organ grafts could act as bridges to allotransplantation.

FUTURE DEVELOPMENTS

Organs, tissues, and cells from gene-edited pigs have great clinical therapeutic potential. Further gene-editing to protect the organ from the human adaptive immune response may include deletion of expression of SLA class 1 [116], or downregulation of SLA class II [117], or expression of PD-L1 [118,119]. This will hopefully enable exogenous immunosuppressive therapy to be significantly reduced or, indeed, ultimately unnecessary.

However, in relation to the adaptive immune response, several questions need investigation. For example, (i) are more peptides presented when transplantation is between different species than within the same species? Whereas in allotransplantation, donor-derived peptides are limited to mismatched HLA, in xenotransplantation any pig protein (not just SLA) that differs in amino acids from human amino acids could be a target. (ii) Do these amino acids activate follicular helper T cells (effector memory T cells)? If the original pig proteins from which the peptides are derived can be identified, gene-editing may allow the pig proteins to be converted to human proteins, thus reducing the strength of the immune response.

The ultimate goal of both allotransplantation and xenotransplantation is the induction of immunologic tolerance, in which the recipient no longer attempts to reject the graft. Although efforts in this respect in xenotransplantation have to date been unsuccessful, in view of the potential offered by genetic engineering of the pig, it would seem it is more likely to be achieved in xenotransplantation than in allotransplantation.

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Ethical consideration

Not applicable.

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