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PERSPECTIVES ON THE OPTIMAL GENETICALLY-ENGINEERED PIG IN 2018 FOR INITIAL CLINICAL TRIALS OF KIDNEY OR HEART XENOTRANSPLANTATION

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Abstract

For a clinical trial today, what might realistically be the optimal pig among those currently available? Deletion of expression of the 3 pig carbohydrate antigens against which humans have natural (preformed) antibodies (triple-knockout [TKO] pigs) should form the basis of any clinical trial. However, because both complement and coagulation can be activated in the absence of antibody, the expression of human complement- and coagulation-regulatory proteins is likely to be important in protecting the graft further. Any genetic manipulation that might reduce inflammation of the graft, eg, expression of hemoxygenase-1 (HO-1) or A20, may also be beneficial to the long-term survival of the graft. The transgene for human CD47 is likely to have a suppressive effect on monocyte/macrophage and T cell activity. Furthermore, deletion of xenoantigen expression, and expression of a human complement-regulatory protein, are both associated with a reduced T cell response. Although there are several other genetic manipulations that may reduce the T cell response further, it seems likely that exogenous immunosuppressive therapy, particularly if it includes costimulation blockade, will be sufficient. We would therefore suggest that, with our present knowledge and capabilities, the optimal pig might be a TKO pig that expressed 1 or more human complement-regulatory proteins, 1 or more human coagulation-regulatory proteins, a human antiinflammatory transgene, and CD47. Absent or minimal antibody binding is important, but we suggest that the additional insertion of protective human transgenes will be beneficial, and may be essential.

Introduction

Experimental organ xenotransplantation has made significant advances during the past few years,^{1–3} and increasing attention is being paid towards initiating clinical trials, particularly

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Disclosure

The authors declare no conflicts of interest.

Authorship

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of pig kidney or heart transplantation,^{4,5} where the pathobiological problems, although slightly different,⁶ are less severe than after liver or lung transplantation. With life-supporting pig kidney transplantation in nonhuman primates (NHPs) extending to beyond 1 year,^{7–10} and heterotopic heart transplantation extending beyond 2 years,^{11–12} in our opinion, it may soon be ethical to move towards a clinical trial.

There are now a variety of genetically-engineered pigs available or potentially available for use as the organ-source for a clinical trial.² Although there will undoubtedly be a greater variety in the future, the question can be asked as to what might realistically be the optimal pig among those currently available or that could become available in the very near future? This question revolves around 2 major genetic manipulations – (i) deletion of expression of xenoantigens against which humans are known to have natural (preformed) antibodies, and (ii) the transgenic expression of human complement- and/or coagulation-regulatory and/or antiinflammatory proteins.^{2,13}

We here put forward our own opinions on what genetically-engineered pigs would be optimal as sources of kidneys or hearts if a clinical trial of xenotransplantation were to be initiated today. We would emphasize that the opinions we provide are personal, and that others in this field of research may have differing opinions. Furthermore, the data we provide have largely been selected to support our opinions.

Deletion of pig antigens against which humans have natural antibodies

There are currently 3 known xenoantigens – (i) galactose- α 1,3-galactose (Gal), a product of the enzyme, α 1,3-galactosyltransferase (GT),^{14,15} (ii) N-glycolylneuraminic acid (Neu5Gc), a product of the enzyme, cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH),^{16–18} and (iii) Sda (a product of the enzyme, β -1,4N-acetylgalactosaminyltransferase).¹⁹

It should be noted that Old World NHPs express Neu5Gc, and therefore make antibodies only against Gal and Sda, whereas humans do not express any of the 3, and therefore make antibodies against all of them. In contrast, New World monkeys do express Gal, but do not express Neu5Gc and Sda, and so make antibodies against these 2 glycans [Hara H et al, unpublished].

Knockout of even one of these, particularly of Gal,^{20,21} reduces in vitro IgM and IgG antibody binding to pig cells significantly (Figure 1),^{22,23} reduces serum cytotoxicity (Figure 2), and extends pig heart or kidney graft survival in immunosuppressed NHP recipients. In the initial studies, heterotopic pig heart graft survival was extended to almost 6 months,^{24,25} and (thymo)kidney graft survival to almost 3 months.²⁶

Antibodies to Gal and Neu5Gc are particularly low in patients with end-stage renal disease undergoing regular hemodialysis (Figure 3), as is antibody binding to pig cells.²⁷ The reason for this is uncertain, but is more likely related to the immunocompromised state associated with renal failure rather than to the dialysis itself, as it is not thought likely that antibody is removed by hemodialysis. Patients undergoing chronic dialysis, therefore, should initially be at low risk of rejecting a pig graft by an antibody-mediated mechanism.

Although, to our knowledge, only one in vivo study using pigs that do not express 2 or 3 of these glycans has been reported,¹⁰ in vitro studies confirm that antibody binding and serum cytotoxicity are reduced further when 2 or all 3 glycans have been deleted from the pig cells.^{28–31}

On this evidence, it seems reasonable to conclude that triple-knockout (TKO) pigs should form the basis of any clinical trial. There are some in the field of xenotransplantation research who feel that this is all that is required. Based on experience in allotransplantation, where the absence of any preformed antibody binding to the graft (and the prevention of the development of de novo antibody by effective immunosuppressive therapy) results in excellent outcomes, it is suggested that an absence of natural antibody should be associated with equally good outcomes after xenotransplantation (as long as the immunosuppressive therapy is totally effective).

However, we suggest that this hypothesis may be false because xenografts are not allografts. The mechanism by which a pig xenograft can be injured is much more complex than rejection of an allograft¹ (Table 1). Although most of the same mechanisms occur after allotransplantation, they are of significantly less magnitude than after xenotransplantation, and some are qualitatively different, eg, coagulation dysregulation. Factors such as complement activation unrelated to antibody binding, molecular incompatibilities that contribute to coagulation dysregulation, the action of innate immune cells, and a systemic inflammatory response may all play a much greater role in destruction of a xenograft than of an allograft. There is increasing evidence that all of these mechanisms can be activated in the absence of antibody. We here (below) draw attention to some of these points.

We have observed one additional benefit of deletion of Gal expression, and that is that it is associated with a reduced T cell response.³² The additional deletion of Neu5Gc expression reduces the T cell response further (Figure 4), and this beneficial effect may be further enhanced if a TKO pig organ is the target for T cells.

Expression of human protective transgenes

Complement activation

Complement can be activated by the classical pathway, in which case an absence of human antibody binding to a pig graft should prevent activation of this pathway. However, complement can also be activated through the alternative or lectin pathways in the absence of antibody binding.³³ Such factors as ischemia-reperfusion injury^{34–41} and an infectious episode^{42–46} may activate complement and cause graft injury. Compared with *human* complement-regulatory proteins (CRPs), *pig* CRPs are relatively ineffective in protecting against *human* complement activation (unless overexpressed⁴⁷), but the introduction into the pig of a *human* complement-regulatory transgene provides much greater protection.^{22,48}

In the very early days of xenotransplantation research, the expression of human (h) CD55 (decay-accelerating factor) extended pig graft survival from minutes, hours, or days to weeks or months.^{49,50} Importantly, in these studies, no steps had been taken to either delete any pig xenoantigens, which in any case was impossible at that time, or reduce recipient NHP

antipig antibody levels. The expression of hCD55 could extend pig kidney graft survival approximately to the same extent as deletion of expression of Gal, fully demonstrating its beneficial effect.

When GTKO pig grafts were transplanted into NHPs, the additional expression of a hCRP reduced the incidence of early graft failure.^{51–53} However, NHP antibody still bound to the GTKO pig cells, and perhaps hCRP expression would not have provided extra protection if there had been *no* antibody binding, as might occur if a TKO graft had been transplanted.

Nevertheless, on the basis of the available evidence, we would suggest that expression of at least 1 hCRP would be beneficial, and certainly not detrimental. Furthermore, Miyagawa has produced evidence suggesting that expression of 2 hCRPs is more effective than 1.^{54,55}

Just as absence of expression of Gal is associated with a reduced T cell response³², expression of a hCRP may similarly be associated with a reduced T cell response.⁵⁶

Coagulation dysfunction

When a GTKO pig organ, with or without expression of a hCRP, was transplanted into a NHP, molecular incompatibilities between the coagulation systems of pig and primate resulted in the development of thrombotic microangiopathy in the graft and consumptive coagulopathy in the recipient. This again may be associated with antibody, or possibly complement, activation of the vascular endothelium of the graft. It thus may not occur if there is *no* antibody activation of the vascular endothelial cells, but this assumption depends entirely on the complete absence of natural antibody in the host and, furthermore, on the absolute efficacy of the immunosuppressive regimen in preventing any *de novo* antibody production. This has proved difficult even in allotransplantation.⁵⁷

It should also be noted that Lin has demonstrated *in vitro* that coagulation can be activated by human platelets and/or innate immune cells, eg, monocytes, in the *absence* of antibody.^{58–60}

In contrast, the expression of a human coagulation-regulatory protein, eg, thrombomodulin, endothelial protein C receptor, or both, delays graft failure significantly.^{8,9,61} Expression of tissue factor pathway inhibitor, CD39, and/or CD73 may also be effective in this respect.

To illustrate this point, we would draw attention to our own experience in which 2 GTKO.hCD46 pig kidney grafts that expressed human coagulation-regulatory proteins functioned well in baboons for >7 and >8 months, respectively, until infectious complications necessitated termination of the experiments, whereas 2 baboons with kidney transplants from GTKO.hCD46 pigs (initially thought to be expressing human thrombomodulin, but later found *not* to be expressing this human coagulation-regulatory protein) developed consumptive coagulopathy within 12 days.⁹ Except for the genetic modifications in the pigs, all other aspects of the experiments, eg, immunosuppressive therapy, were identical.

This study also demonstrated that, even when a low level of serum antipig antibody was present in the 2 long-surviving baboons, graft rejection could be prevented by the expression

of the human transgenes. This would be a safeguard in patients in whom a low level of antibody was erroneously not detected or in whom a low level of de novo antibody develops.

Any thrombin activation can be detrimental to the outcome of the transplant. For example, thrombin activates the human cellular response to pig cells in vitro (Figure 5).⁶² This was an important observation as it indicates that cellular rejection can be stimulated by mechanisms that are not considered part of the adaptive immune response.

Systemic inflammation

A systemic inflammatory response has been well-described after pig organ (and even artery patch) transplantation in NHPs, and is illustrated by a sustained (>3 months) rise in C-reactive protein in the recipient (Figure 6).^{63–66} There is evidence that it precedes the onset of disturbances in coagulation (Figure 7).^{63,64} The inflammatory response is associated with deposition of C-reactive protein in the transplanted pig kidney (Figure 8). Whether this is related to antibody binding to the graft remains uncertain. However, it is well-known that inflammation and the innate immune response augment the adaptive immune response to a graft (Figure 9), and contribute to activation of coagulation dysfunction,^{62–64,67,68} and therefore any genetic manipulation that might reduce inflammation of the graft, eg, expression of hemeoxygenase-1 (HO-1) or A20, may be beneficial to the long-term survival of the graft.

There is little or no rise in C-reactive protein in allotransplantation models in NHPs,^{63,64} perhaps supporting our hypothesis that the pathobiological response to a xenograft is different from that to an allograft, although this could be related to an absence of antibody binding to the allograft vascular endothelium.

Although the administration of an antiinflammatory agent, eg, tocilizumab, to the recipient of the graft may suppress the systemic inflammatory response (as evidenced by reduced levels of C-reactive protein or serum amyloid A),^{65,66,69} recent evidence indicates that tocilizumab, although binding to primate IL-6 receptors, does not bind to IL-6 receptors on the pig graft⁶⁷ (Gao H et al, manuscript submitted; Hara H et al, unpublished data), and therefore has no protective effect on the graft.

An important observation made recently by our group indicates that, when pig vascular endothelial cells expressing only natural pig thrombomodulin (which also has an antiinflammatory effect) are activated by tumor necrosis factor-alpha (TNF- α), the expression of thrombomodulin is significantly downregulated (Figure 10).⁶⁸ This suggests that, when a pig organ is exposed to inflammation (which we have documented is *universal* after a pig organ transplant into a NHP), thrombotic microangiopathy is likely to develop. In contrast, in pig vascular endothelial cells transgenic for human thrombomodulin, thrombomodulin is *not* downregulated when the cells are activated by TNF- α , but is significantly upregulated (Figure 10). We presume that expression of the transgenically-expressed human thrombomodulin remains stable as a result of how the transgene was introduced (in this case using an ICAM-2 promoter), though the exact mechanism remains uncertain. Nevertheless, this benefit from the transgenic procedure may be a major reason why pig heart transplants have performed so well after transplantation in NHPs,¹² and why

the absence of the antiinflammatory effect of a human transgene, such as thrombomodulin, resulted in the early development of consumptive coagulopathy in the recipient of the pig kidney graft (reviewed above).⁹

Comment

One topic not discussed above is that of expression of swine leukocyte antigens (SLA) on the graft. Although little work has been carried out to explore this topic in NHPs, immunologically-naïve NHPs do not appear to have antibodies directed against SLA. However, there is increasing evidence that at least some patients with anti-HLA antibodies, ie, with panel-reactive antibodies, may have antibodies that cross-react with SLA expressed on the pig graft.^{23,70,71} Although genetic engineering techniques are being developed to overcome this problem, largely by Tector and his colleagues,^{70–72} in the initial clinical trials it might be wise to exclude any patients with anti-HLA antibodies.

As the absence of antibody binding to the graft is of such fundamental importance, TKO pigs will be the basic sources of organs for clinical xenotransplantation. However, it would seem only sensible, if not mandatory, for the graft to express several human transgenes for which there is considerable in vivo and/or in vitro evidence of benefit. These include both hCRPs and human coagulation-regulatory proteins, some of which also have antiinflammatory functions, and transgenes such as HO-1 that has an antiinflammatory protective effect. Furthermore, the introduction of human CD47 has a suppressive effect on the function of monocytes and macrophages and also on T cell activity,^{73,74} and the expression of HLA-E or HLA-G may also suppress innate cell function, particularly of natural killer (NK) cells.^{75–78} A TKO pig expressing CD46, CD55, thrombomodulin and EPCR would be close to optimal, and the additional expression of HO-1 and/or CD47 would likely be beneficial. It is very unlikely that any group will be able to assess in vivo the importance of each of the 9 genetic modifications individually, but it is hoped that national regulatory authorities will accept in vitro evidence that indicates the benefit of each deletion and transgene.⁵

The above genetic engineering approach is directed mainly to overcoming the innate immune response, which has been most problematic to date. It thus relies on the administration of immunosuppressive agents to the recipient NHP (or human patient) to prevent an adaptive immune response. In this respect, agents that block the CD40/CD154 costimulation pathway have proved most successful.^{7–9,11,12,24–26,79–81}

However, additional genetic manipulations that reduce the T cell response, eg, expression of a mutant SLA class II transactivator (CIITA) gene (that results in reduced expression of SLA class II),^{82,83} knockout of SLA class I,⁷² or expression of CD47,^{73,74} are all likely to be beneficial in allowing the intensity of exogenous immunosuppressive therapy to be reduced. Alternatively, or additionally, human CTLA4-Ig can be expressed in the pig organ.⁸⁴ This would suppress both the direct and indirect T cell responses, and produce a local immunosuppressive effect. In contrast, pig CTLA4-Ig is less effective at binding to human CD80/86.⁸⁵

In conclusion, absent or minimal antibody binding is clearly important, but the ability to protect the graft further by insertion of selected human transgenes will almost certainly provide additional protection.

We have not commented on genetic manipulations that can be made to prevent porcine endogenous retrovirus (PERV) activation, in part because we are not convinced that this is necessary. However, if proved essential, activation of PERV can be inhibited^{86–88} or PERV can be deleted⁸⁹. Whether deletion of 62 PERV genes, in addition to the genetic manipulations discussed above, will affect the viability or function of the pig cells remains uncertain.

Finally, although pig kidneys and heart have functioned satisfactorily for months after transplantation into NHPs, genetic manipulations may prove beneficial to resolve any functional discrepancies between pig and primate, if these are identified. For example, if remains uncertain whether porcine erythropoietin functions satisfactorily in primates, but, if this proves to be the case, the introduction of the transgene for human erythropoietin may be required. This topic and other physiological aspects of kidney xenotransplantation have recently been discussed elsewhere.⁹⁰

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Abbreviations

CMAH-KO	cytidine monophosphate-N-acetylneuraminic acid hydroxylase gene-Knockout
CRP	complement-regulatory protein
EPCR	endothelial protein C receptor
Gal	galactose- α 1,3-galactose
GTKO	α 1,3-galactosyltransferase gene-knockout
hCRP	human complement-regulatory protein
HLA	human leukocyte antigen
Neu5Gc	N-glycolylneuraminic acid
NHP	nonhuman primate
SLA	swine leukocyte antigen
TKO	triple-knockout (pigs that do not express the 3 known pig xenoantigens, Gal, Neu5Gc, or Sda)

References

1. Cooper DKC, Ezzelarab MB, Hara H, et al. The pathobiology of pig-to-primate xenotransplantation: a historical review. *Xenotransplantation*. 2016;23:83–105. [PubMed: 26813438]
2. Cooper DKC, Ekser B, Ramsoondar J, Phelps C, Ayares D. The role of genetically-engineered pigs in xenotransplantation research. *J Pathol*. 2016;238:288–299. [PubMed: 26365762]
3. Cooper DKC, Gaston R, Eckhoff D, et al. Xenotransplantation - current status and prospects. *Br Med Bull*. 2018;125:5–14. [PubMed: 29228112]
4. Cooper DKC, Wijkstrom M, Hariharan S, et al. Selection of patients for initial clinical trials of solid organ xenotransplantation. *Transplantation*. 2017;101:1551–1558. [PubMed: 27906824] ;:
5. Cooper DKC, Pierson RN, III, Hering BJ, et al. Regulation of clinical xenotransplantation – time for a reappraisal?. *Transplantation*. 2017;102:1766–1769.
6. Knosalla C, Yazawa K, Behdad A, et al. Renal and cardiac endothelial heterogeneity impact acute vascular rejection in pig-to-baboon xenotransplantation. *Am J Transplant*. 2009;9:1006–1016. [PubMed: 19422330]
7. Higginbotham L, Mathews D, Breeden CA, et al. Pre-transplant antibody screening and anti-CD154 costimulation blockade promote long-term xenograft survival in a pig-to-primate kidney transplant model. *Xenotransplantation*. 2015;22:221–230. [PubMed: 25847130]
8. Iwase H, Liu H, Wijkstrom M, et al. Pig kidney graft survival in a baboon for 136 days: longest life-supporting organ graft survival to date. *Xenotransplantation*. 2015;22:302–309. [PubMed: 26130164]
9. Iwase H, Hara H, Ezzelarab M, et al. Immunological and physiological observations in baboons with life-supporting genetically engineered pig kidney grafts. *Xenotransplantation*. 2017;24(2).
10. Martens G, Estrada J, Sidner RA, et al. Porcine GGTA1/B4GalNT2 gene knockout reduces antibody binding and achieves one year life-supporting renal xenograft in pig-to-rhesus model. *Xenotransplantation*. 2017;24:12–15. (Abstract) (PL3.4).
11. Mohiuddin MM, Singh AK, Corcoran PC, et al. One-year heterotopic cardiac xenograft survival in a pig to baboon model. *Am J Transplant*. 2014;14:488–489. [PubMed: 24330419]
12. Mohiuddin MM, Singh AK, Corcoran PC, et al. Chimeric 2C10R4 anti-CD40 antibody therapy is critical for long-term survival of GTKO.hCD46.hTBM pig-to-primate cardiac xenograft. *Nat Commun*. 2016;7:11138. [PubMed: 27045379]
13. Butler JR, Ladowski JM, Martens GR, Tector M, Tector AJ. Recent advances in genome editing and creation of genetically modified pigs. *Int J Surg*. 2015;23:217–222. [PubMed: 26231992]
14. Good AH, Cooper DKC, Malcolm AJ, et al. Identification of carbohydrate structures that bind human antiporcine antibodies: implications for discordant xenografting in man. *Transplant Proc*. 1992;24:559–562. [PubMed: 1566430]
15. Cooper DKC. Depletion of natural antibodies in non-human primates - a step towards successful discordant xenografting in humans. *Clin Transplantation*. 1992;6:178–183.
16. Bouhours D, Pourcel C, Bouhours JE. Simultaneous expression by porcine aorta endothelial cells of glycosphingolipids bearing the major epitope for human xenoreactive antibodies (Gal alpha1–3Gal), blood group H determinant and N-glycolylneuraminic acid. *Glycoconj J*. 1996;13:947–953. [PubMed: 8981086]
17. Zhu A, Hurst R. Anti-N-glycolylneuraminic acid antibodies identified in healthy human serum. *Xenotransplantation*. 2002;9:376–381. [PubMed: 12371933]
18. Padler-Karavani V, Varki A. Potential impact of the non-human sialic acid n-glycolylneuraminic acid on transplant rejection risk. *Xenotransplantation*. 2011;18:1–5. [PubMed: 21342282]
19. Byrne GW, Du Z, Stalboerger P, Kogelberg H, McGregor CG. Cloning and expression of porcine beta1,4 n-acetylgalactosaminyl transferase encoding a new xenoreactive antigen. *Xenotransplantation*. 2014;21:543–554. [PubMed: 25176027]
20. Phelps CJ, Koike C, Vaught TD, et al. Production of alpha 1,3-galactosyltransferase-deficient pigs. *Science (New York, NY)*. 2003;299:411–414.

21. Kolber-Simonds D, Lai L, Watt SR, et al. Production of alpha-1,3-galactosyltransferase null pigs by means of nuclear transfer with fibroblasts bearing loss of heterozygosity mutations. *Proc Natl Acad Sci U S A*. 2004;101:7335–7340. [PubMed: 15123792]
22. Hara H, Long C, Lin YJ, et al. In vitro investigation of pig cells for resistance to human antibody-mediated rejection. *Transpl Int*. 2008;21:1163–1174. [PubMed: 18764834]
23. Martens GR, Reyes LM, Butler JR, et al. Humoral reactivity of renal transplant-waitlisted patients to cells from GGTA1/CMAH/B4GalNT2, and SLA class I knockout pigs. *Transplantation*. 2017;101:e86–e92. [PubMed: 28114170]
24. Kuwaki K, Tseng YL, Dor FJ, et al. Heart transplantation in baboons using α 1,3-galactosyltransferase gene-knockout pigs as donors: initial experience. *Nat Med*. 2005;11:29–31. [PubMed: 15619628]
25. Tseng YL, Kuwaki K, Dor FJ, et al. α 1,3-galactosyltransferase gene-knockout pig heart transplantation in baboons with survival approaching six months. *Transplantation*. 2005;80:1493–1500. [PubMed: 16340796]
26. Yamada K, Yazawa K, Shimizu A, et al. Marked prolongation of porcine renal xenograft survival in baboons through the use of α 1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. *Nat Med*. 2005;11:32–34. [PubMed: 15619627]
27. Zhang Z, Hara Z, Long C, et al. Immune responses of HLA-highly-sensitized and non-sensitized patients to genetically-engineered pig cells. *Transplantation*. 2018;102:e195–e204. [PubMed: 29266033]
28. Burlak C, Paris LL, Lutz AJ, et al. Reduced binding of human antibodies to cells from GGTA1/CMAH KO pigs. *Am J Transplant*. 2014;14:1895–1900. [PubMed: 24909344]
29. Lutz AJ, Li P, Estrada JL, et al. Double knockout pigs deficient in N-glycolylneuraminic acid and galactose α -1,3-galactose reduce the humoral barrier to xenotransplantation. *Xenotransplantation*. 2013;20:27–35. [PubMed: 23384142]
30. Lee W, Hara H, Ezzelarab MB, et al. Initial in vitro studies on tissues and cells from GTKO/hCD46/NeuGcKO pigs. *Xenotransplantation*. 2016;23:137–150. [PubMed: 26988899]
31. Estrada JL, Martens G, Li P, Adams A, et al. Evaluation of human and non-human primate antibody binding to pig cells lacking GGTA1/CMAH/beta4GALNT2 genes. *Xenotransplantation*. 2015;22:194–202. [PubMed: 25728481]
32. Wilhite T, Ezzelarab C, Hara H, et al. The effect of Gal expression on pig cells on the human T-cell xenoreponse. *Xenotransplantation*. 2012;19:56–63. [PubMed: 22360754]
33. Zhou H, Wang L, Hara H, Dalmaso A, Cooper DKC. The complex functioning of the complement system in xenotransplantation. 2018 Submitted.
34. Danobeitia JS, Ziemelis M, Ma X, et al. Complement inhibition attenuates acute kidney injury after ischemia-reperfusion and limits progression to renal fibrosis in mice. *PLoS One*. 2017;12:e0183701. [PubMed: 28832655]
35. Grenda R, Durlik M. Eculizumab in Renal Transplantation: A 2017 Update. *Ann Transplant*. 2017;22:550–554. [PubMed: 28894081]
36. Legendre C, Sberro-Soussan R, Zuber J, Frémeaux-Bacchi V. The role of complement inhibition in kidney transplantation. *Br Med Bull* 2017;124:5–17. [PubMed: 29069327]
37. Nauser CL, Farrar CA, Sacks SH. Complement Recognition Pathways in Renal Transplantation. *J Am Soc Nephrol*. 2017;28:2571–2578. [PubMed: 28663231]
38. Zhang K, Li GQ H, et al. C5a/C5aR pathway accelerates renal ischemia-reperfusion injury by downregulating PGRN expression. *Int Immunopharmacol*. 2017;53:17–23. [PubMed: 29031143]
39. Zwaini Z, Dai H, Stover C, Yang B. Role of Complement Properdin in Renal Ischemia-Reperfusion Injury. *Curr Gene Ther*. 2017;17:411–423. [PubMed: 29446739]
40. Jordan SC, Choi J, Aubert O, et al. A Phase I/II, Double-Blind, Placebo-Controlled Study Assessing Safety and Efficacy of C1 Esterase Inhibitor for Prevention of Delayed Graft Function in Deceased Donor Kidney Transplant Recipients. *Am J Transplant*. Published online April 10, 2018. DOI:10.1111/ajt.14767.
41. Kaabak M, Babenko N, Shapiro R, Zokoyev A, Dymova O, Kim E. A prospective randomized, controlled trial of eculizumab to prevent ischemia-reperfusion injury in pediatric kidney transplantation. *Pediatr Transplant*. 2018;22(2).

42. Skattum L, van Deuren M, van der Poll T, Truedsson L. Complement deficiency states and associated infections. *Mol Immunol*. 2011;48:1643–1655. [PubMed: 21624663]
43. Ricklin D, Lambris JD. Complement in immune and inflammatory disorders: pathophysiological mechanisms. *J Immunol*. 2013;190:3831–3838. [PubMed: 23564577]
44. Lewis LA, Ram S. Meningococcal disease and the complement system. *Virulence*. 2014;5:98–126. [PubMed: 24104403]
45. Napier BA, Brubaker SW, Sweeney TE, et al. Complement pathway amplifies caspase-11-dependent cell death and endotoxin-induced sepsis severity. *J Exp Med*. 2016;213:2365–2382. [PubMed: 27697835]
46. Kuusela P, Saraswat M, Joenväärä S, Kaartinen J, Järvinen A, Renkonen R. Changes in plasma protein levels as an early indication of a bloodstream infection. *PLoS One*. 2017;12:e0172987. [PubMed: 28235076]
47. Morgan BP, Berg CW, Harris CL. “Homologous restriction” in complement lysis: roles of membrane complement regulators. *Xenotransplantation* 2005;12:258–265. [PubMed: 15943774]
48. Cozzi E, White DJ. The generation of transgenic pigs as potential organ donors for humans. *Nat Med*. 1995;1:964–966. [PubMed: 7585226]
49. Cozzi E, Vial C, Ostlie D, et al. Maintenance triple immunosuppression with cyclosporin A, mycophenolate sodium and steroids allows prolonged survival of primate recipients of hDAF porcine renal xenografts. *Xenotransplantation*. 2003;10:300–310. [PubMed: 12795679]
50. Baldan N, Rigotti P, Calabrese F, et al. Ureteral stenosis in HDAF pig-to-primate renal xenotransplantation: a phenomenon related to immunological events? *Am J Transplant*. 2004;4:475–481. [PubMed: 15023139]
51. McGregor CG, Ricci D, Miyagi N, et al. Human CD55 expression blocks hyperacute rejection and restricts complement activation in Gal knockout cardiac xenografts. *Transplantation*. 2012;93:686–692. [PubMed: 22391577]
52. Azimzadeh A, Kelishadi S, Ezzelarab M, et al. Early graft failure of gtko pig organs in baboons is reduced in hcrp expression. *Xenotransplantation*. 2009;16:356 (Abstract).
53. Azimzadeh AM, Kelishadi SS, Ezzelarab MB, et al. Early graft failure of GalTKO pig organs in baboons is reduced by expression of a human complement pathway-regulatory protein. *Xenotransplantation*. 2015;22:310–316. [PubMed: 26174749]
54. Miyagawa S, Shirakura R, Iwata K, et al. Effects of transfected complement regulatory proteins, MCP, DAF, and MCP.DAF hybrid, on complement-mediated swine endothelial cell lysis. *Transplantation*. 1994;58:834–840. [PubMed: 7524206]
55. Miyagawa S, Yamamoto A, Matsunami K, et al. Complement regulation in the GalT KO era. *Xenotransplantation*. 2010;17:11–25. [PubMed: 20149185]
56. Ezzelarab MB, Ayares D, Cooper DKC. Transgenic expression of human CD46: does it reduce the primate T-cell response to pig endothelial cells? *Xenotransplantation*. 2015; 22:487–489. [PubMed: 26584837]
57. Everly MJ, Rebellato LM, Haisch CE, et al. Incidence and impact of de novo donor-specific alloantibody in primary renal allografts. *Transplantation*. 2013;95:410–417. [PubMed: 23380861]
58. Lin CC, Chen D, McVey JH, Cooper DKC, Dorling A. Expression of tissue factor and initiation of clotting by human platelets and monocytes after incubation with porcine endothelial cells. *Transplantation*. 2008;86:702–709. [PubMed: 18791452]
59. Lin CC, Ezzelarab M, Shapiro R, et al. Recipient tissue factor expression is associated with consumptive coagulopathy in pig-to-primate kidney xenotransplantation. *Am J Transplant*. 2010;10:1556–1568. [PubMed: 20642682]
60. Ezzelarab MB, Liu YW, Lin CC, et al. Role of P-selectin and P-selectin glycoprotein ligand-1 interaction in the induction of tissue factor expression on human platelets after incubation with porcine aortic endothelial cells. *Xenotransplantation*. 2014;21:16–24. [PubMed: 25405250]
61. Iwase H, Ekser B, Satyananda V, et al. Pig-to-baboon heterotopic heart transplantation – exploratory preliminary experience with pigs transgenic for human thrombomodulin and comparison of three costimulation blockade-based regimens. *Xenotransplantation*. 2015;22:211–220. [PubMed: 25847282]

62. Ezzelarab C, Ayares D, Cooper DKC, Ezzelarab MB. Human T-cell proliferation in response to thrombin-activated GTKO pig endothelial cells. *Xenotransplantation*. 2012;19:311–316. [PubMed: 22970807]
63. Ezzelarab MB, Ekser B, Azimzadeh A, et al. Systemic inflammation in xenograft recipients precedes activation of coagulation. *Xenotransplantation*. 2015;22:32–47. [PubMed: 25209710]
64. Ezzelarab MB, Cooper DKC. Systemic inflammation in xenograft recipients (SIXR): A new paradigm in pig-to-primate xenotransplantation? *Int J Surg*. 2015;23(Pt B):301–305. [PubMed: 26209584]
65. Iwase H, Ekser B, Zhou H, et al. Further evidence for sustained systemic inflammation in xenograft recipients (SIXR). *Xenotransplantation*. 2015;22:399–405. [PubMed: 26292982]
66. Iwase H, Liu H, Li T, et al. Therapeutic regulation of systemic inflammation in xenograft recipients. *Xenotransplantation*. 2017;24(2).
67. Gao H, Lu L, Zhao Y, et al. Human IL-6, IL-17, IL-1 β and TNF- α differently regulate the expression of pro-inflammatory related genes, tissue factor, and swine leukocyte antigen class I in porcine aortic endothelial cells. *Xenotransplantation*. 2017;24(2).
68. Hara H, Iwase H, Miyagawa Y, et al. Reducing the inflammatory response by expressing human thrombomodulin in pigs. *Xenotransplantation*. 2017;24:19–20. (Abstract) (PL.3.11).
69. Li T, Iwase H, Hara H, Long C, et al. Serum amyloid A as a marker of inflammation in xenotransplantation. *Eur J Inflamm*. 2018 Submitted.
70. Ladowski JM, Martens GR, Reyes LM, et al. Examining the biosynthesis and xenoantigenicity of class II swine leukocyte antigen protein. *J Immunol*. 2018;200:2957–2964. [PubMed: 29540579]
71. Ladowski JM, Reyes LM, Martens GR, et al. Swine leukocyte antigen class II is a xenoantigen. *Transplantation*. 2018;102:248–254.
72. Reyes LM, Estrada JL, Wang ZY, et al. Creating class I MHC-null pigs using guide RNA and the Cas9 endonuclease. *J Immunol*. 2014;193:5751–5757. [PubMed: 25339675]
73. Tena A, Kurtz J, Leonard DA, et al. Transgenic expression of human CD47 markedly increases engraftment in a murine model of pig-to-human hematopoietic cell transplantation. *Am J Transplant*. 2014;14:2713–2722. [PubMed: 25278264]
74. Tena AA, Sachs DH, Mallard C, et al. Prolonged survival of pig skin on baboons after administration of pig cells expressing human CD47. *Transplantation*. 2017;101:316–321. [PubMed: 27232934]
75. Sasaki H, Xu XC, Smith DM, Howard T, Mohanakumar T. HLA-G expression protects porcine endothelial cells against natural killer cell-mediated xenogeneic cytotoxicity. *Transplantation*. 1999;67:31–37. [PubMed: 9921792]
76. Dorling A, Monk N, Lechler R. HLA-G inhibits the transendothelial cell migration of human NK cells: A strategy for inhibiting xenograft rejection. *Transplant Proc*. 2000;32:938. [PubMed: 10936283]
77. Forte P, Baumann BC, Weiss EH, Seebach JD. HLA-E expression on porcine cells: Protection from human NK cytotoxicity depends on peptide loading. *Am J Transplant*. 2005;5:2085–2093. [PubMed: 16095487]
78. Crew MD. Play it in E or G. Utilization of HLA-E and -G in xenotransplantation. *Xenotransplantation*. 2007;14:198–207. [PubMed: 17489859]
79. Bühler L, Awwad M, Basker M, et al. High-dose porcine hematopoietic cell transplantation combined with CD40 ligand blockade in baboons prevents an induced anti-pig humoral response. *Transplantation*. 2000;69:2296–2304. [PubMed: 10868629]
80. Ezzelarab M, Garcia B, Azimzadeh A, et al. The innate immune response and activation of coagulation in α 1,3-galactosyltransferase gene-knockout xenograft recipients. *Transplantation*. 2009;87:805–812. [PubMed: 19300181]
81. Ezzelarab MB, Ekser B, Echeverri G, et al. Costimulation blockade in pig artery patch xenotransplantation - a simple model to monitor the adaptive immune response in nonhuman primates. *Xenotransplantation*. 2012;19:221–232. [PubMed: 22909135]
82. Hara H, Witt W, Crossley T, et al. Human dominant-negative class II transactivator transgenic pigs – effect on the human anti-pig T cell immune response and immune status. *Immunology*. 2013;140:39–46. [PubMed: 23566228]

83. Iwase H, Ekser B, Satyananda V, et al. Initial in vivo experience of pig artery patch transplantation in baboons using mutant MHC (CIITA-DN) pigs. *Transpl Immunol.* 2015; 32:99–108. [PubMed: 25687023]
84. Phelps CJ, Ball SF, Vaught TD, et al. Production and characterization of transgenic pigs expressing porcine CTLA4-Ig. *Xenotransplantation.* 2009;16:477–485. [PubMed: 20042047]
85. Koshika T, Phelps C, Fang J, et al. Relative efficiency of porcine and human cytotoxic T-lymphocyte antigen 4 immunoglobulin in inhibiting human CD4+ T-cell responses co-stimulated by porcine and human B7 molecules. *Immunology.* 2011;134:386–97. [PubMed: 22043861]
86. Dieckhoff B, Karlas A, Hofmann A, et al. Inhibition of porcine endogenous retroviruses (PERVs) in primary porcine cells by RNA interference using lentiviral vectors. *Arch Virol.* 2007;152:629–634. [PubMed: 17106623]
87. Dieckhoff B, Petersen B, Kues WA, et al. Knockdown of porcine endogenous retrovirus (PERV) expression by PERV-specific shRNA in transgenic pigs. *Xenotransplantation.* 2008;15:36–45. [PubMed: 18333912]
88. Ramsoondar J, Vaught T, Ball S, et al. Production of transgenic pigs that express porcine endogenous retrovirus small interfering RNAs. *Xenotransplantation* 2009;16:164–180. [PubMed: 19566656]
89. Niu D, et al., Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. *Science.* 2017;357:1303–1307. [PubMed: 28798043]
90. Iwase H, Edwin E, Cooper DKC. Physiological aspects of pig kidney transplantation in primates. AALAS-CM. 2018 In press.

Figure 1: Human serum IgM (left) and IgG (right) binding to red blood cells (RBCs).

RBCs were drawn from (i) a volunteer healthy human, (ii) a wild-type (WT) pig, (iii) an α 1,3-galactosyltransferase gene-knockout (GTKO) pig, (iv) a GTKO pig that did not express Sda (GTKO/ β 4GalKO or double-knockout [DKO]), (iv) a DKO pig that did not express Neu5Gc (GTKO/ β 4GalKO/CMAHKO or triple-knockout [TKO] pig). Human RBCs (O blood type) were used as a negative control. Fourteen human sera were tested for antibody binding to pRBCs, and 6 human sera were tested for antibody binding to hRBCs. Human IgM and IgG binding to GTKO/ β 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. Relative GM = relative geometric mean, which was calculated by dividing the geometric mean value for each sample by the negative control (secondary antibody only). ns=not significant; **P<0.01. (For details of methods, see reference 67.)

Figure 2: Protection of pig aortic endothelial cells (pAEC) from human serum cytotoxicity by transgenic expression of a human complement-regulatory protein, CD46.

The pAEC tested included (left-to-right) wild-type (WT, ie, genetically-*un*modified), GTKO, CD46-transgenic, and GTKO/CD46. Activated pAEC were exposed to IFN- γ for 48h. Nonactivated (white bars) and activated (shaded bars) cells were then exposed to 25% human serum. Activation increased lysis of each cell type except GTKO/CD46 cells, which were completely resistant to serum cytotoxicity. (Reproduced with permission from Hara H, et al. *Transpl Int*. 2008;21:1163–1174; reference 22).

Figure 3: Anti-Gal and anti-Neu5Gc IgM and IgG antibody levels in patients with end-stage renal disease (ESRD).

Anti-Gal and anti-Neu5Gc IgM/IgG were measured by ELISA in patients with ESRD (on hemodialysis) who had either (i) a high level (100%) of panel-reactive antibodies (cPRA, ie, antibodies against HLA; n=10), or (ii) a negative cPRA, (ie, 0% anti-HLA antibodies n=12), and in healthy human control subjects (n= 10). The patients with ESRD, whether highly sensitized to HLA or not, had significantly lower levels of anti-Gal and anti-Neu5Gc IgM (top panels) and IgG (bottom panels) antibodies than healthy control subjects. (ns=not significant; *P<0.05, **P<0.01, ***P<0.005, ****P<0.001). (Reproduced with permission from Zhang Z, et al. *Transplantation*. 2018;102:e195-e204; reference 27).

Figure 4: Proliferative response of human T cells (CD3⁺, CD4⁺, and CD8⁺ cells) to wild-type (WT), GTKO/CD46, and GTKO/CD46/CMAHKO pig peripheral blood mononuclear cells (PBMCs).

PBMCs were isolated from healthy human volunteers (n=9). The proliferation of CD3⁺, CD4⁺, and CD8⁺ T cells in human PBMC to pig PBMCs were identified and quantified by flow cytometry at the end of a 5-day CFSE-MLR. Deletion of Gal expression on the pig cells significantly reduced the proliferative response, and deletion of both Gal and Neu5Gc reduced it further. (*P<0.05, **P<0.01). (For details of methods, see reference 27.)

Figure 5: Thrombin activates the human cellular response to GTKO pig peripheral blood mononuclear cells (PBMCs).

(Upper panel) The degree of activation of GTKO pig PBMCs by thrombin was comparable to that resulting from stimulation of the cells by porcine interferon-gamma (pIFN- γ). Thrombin-stimulated activation of the human cellular response was reduced by the addition of hirudin, confirming that thrombin was the stimulatory factor. (Lower panel) In thrombin-activated pig aortic endothelial cells, CD86 (costimulation pathway) mRNA was upregulated in a dose-dependent manner, though this upregulation was reduced by hirudin. (Modified from Ezzelarab C, et al. *Xenotransplantation*. 2012;19:311–316; reference 62).

Figure 6: Response of serum C-reactive protein to the presence of a GTKO/CD46 pig artery patch grafts sutured into the wall of the abdominal aorta of the recipient baboons. After the transplant, there was an immediate and sustained increase (for >3 months) in C-reactive protein, even when the recipient received therapy with either cobra venom factor (CVF) (pretransplantation) to deplete complement, or alpha-1-antitrypsin (AAT) (pre and posttransplantation). However, pre and posttransplant treatment with the IL-6-receptor blockade agent, tocilizumab (Actemra), prevented the rise in C-reactive protein. (For details, see reference 65.)

Figure 7: After pig kidney transplantation in baboons, C-reactive protein (C-RP) increases before the development of a consumptive coagulopathy.

Platelet counts, and fold increases in C-RP and D-dimer were calculated in a baboon recipient of a pig renal xenograft. High levels of C-RP were detected as early as 3 days after kidney xenotransplantation, prior to the development of consumptive coagulopathy (CC), as indicated by reduced platelet counts and elevated D-dimer. (Reproduced with permission from Ezzelarab MB, Cooper DKC. *Int J Surg.* 2015;23:301–305; reference 64.)

Figure 8: C-reactive protein (C-RP) deposition in a pig kidney transplanted into a baboon, an indicator of the inflammatory response to the graft.

(Left panel) At 30 minutes after reperfusion, no C-RP deposition was detected. In 2 different kidneys at the time of euthanasia (middle and right panels), C-RP deposition was detected in the glomeruli (arrow heads, right panel) and tubules (arrows, middle and right panels). Our data suggested that both the xenograft and the recipient contributed to the C-RP production. (We could detect minimal C-RP in NHPs undergoing heart allotransplantation [not shown], illustrating the difference in response to a xenograft from an allograft.) (Reproduced with permission from Ezzelarab MB, et al. *Xenotransplantation*. 2015;22:32–47; reference 63.)

Figure 9: Inflammation increases the proliferative response of human peripheral blood mononuclear cells (PBMC) to WT and GTKO pig aortic endothelial cells (pAEC). In vitro study of the effect of inflammation (exposure to interferon-gamma [IFN]) on the human PBMC response to pAECs. When nonactivated (by IFN), the response to WT pAECs was greater than to GTKO cells ($p<0.05$). There was a significant increase in the PBMC response when the pAEC were activated by IFN ($p<0.01$), the response to WT cells again being significantly greater than to GTKO cells ($p<0.05$). This study illustrates how inflammation can greatly increase the immune response to a xenograft. (CPM=counts per minute; SI=stimulation index). (Reproduced with permission from Wilhite T, et al. *Xenotransplantation*. 2012;19:56–63; reference 32.)

Figure 10: Inflammation down-regulates expression of natural pig thrombomodulin (TBM), but not of transgenic human TBM.

Transgenic expression of human TBM (right), but not of natural pig TBM (left), on pig vascular endothelial cells protects against inflammation. The expression of natural pig TBM was down-regulated after exposure to TNF- α confirmed by real-time PCR (* $P < 0.05$, ** $P < 0.01$), whereas the expression of transgenic human TBM was upregulated confirmed by flow cytometry. Transgenically-expressed human TBM would appear to be resistant to down-regulation by inflammation. (The expression of pig TBM in GTKO/CD46 pig aortic endothelial cells [PAECs] and human TBM in human TBM- transgenic PAECs was measured by real-time PCR and flow cytometry [clone 1A4, BD Biosciences, San Jose, CA], respectively. The PCR primers sequences used were: pTBM: Sense 5' - GAA GCT ATG AGG TCC AGC CC -3'; Antisense 5' - CAG ACA GAC AGC GAA GAG CA -3'.) (Details in Lee W, et al. *Ocul Immunol Inflamm.* 2016;24:579–593).

Table 1:

Factors involved in pig organ graft failure after xenotransplantation *

1	Natural antipig IgM and IgG antibody *
2	Complement activation
3	Coagulation dysfunction
4	Innate immune cells, eg, monocytes/macrophages, neutrophils, natural killer (NK) cells
5	Systemic inflammation
6	T and B cells (elicited antibodies, cellular response)

* There is increasing evidence that all of these mechanisms (with the exception of [1]) can be activated in the absence of antibody.