# Mechanism and Regulation of the Complement Activity in Kidney Xenotransplantation

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**Abstract**

Xenotransplantation is emerging as one of several potential solutions for addressing organ donor shortages, with significant progress bringing it closer to clinical application. However, challenges remain, particularly concerning complement system dysregulation caused by species differences, as well as xenoantigens and coagulopathy. Complement regulatory proteins expressed on endothelial cells of donor xenografts are less compatible with complement components in recipients. These difficulties contribute to hyperacute rejection, characterized by antibody-mediated complement activation that destroys the graft within 24 hours. Moreover, because molecules are incompatible across different species, ischemia-reperfusion injury or infection can easily elicit complement activity via all three pathways, resulting in xenograft loss via complement-mediated vascular injury. Complement activity also stimulate innate and adaptive immune cells. To address this issue, genetic modifications in donor pigs and the development of novel medicines have been tested in preclinical models with promising results. Pigs modified to express human complement-regulating molecules such as CD46, CD55, and CD59 have shown longer kidney xenograft survivals over years in preclinical models with nonhuman primates, paving the way for clinical trials. Anti-complement component agents such as C1 esterase and C5 inhibitors have also been shown to increase xenograft survivals. This review examines the role of the complement system in kidney xenotransplantation, emphasizing new research and clinical trial advancements.

**Key words** (maximum 6): complement, innate immunity, kidney transplantation, xenotransplantation

## 1. Introduction

Xenotransplantation is considered to be one of several potential solutions for donor organ shortages. Although significant progression has been made recently, several concerns must be resolved before clinical use. One of the challenges to overcome is dysregulation of the complement system, which is caused by differences between species as well as xenoantigens and coagulopathy hyperactivity [1,2]. Dysregulation and incompatibility produce hyper acute rejection (HAR), commonly characterized as antibody-mediated complement activation that leads to destruction of the graft within 24 h [3,4]. To address these limitations, in addition to novel immunosuppressive regimens, numerous types of gene-modifications have been attempted in donor pigs, as have new developments in genetic engineering such as clustered regulatory interspaced palindromic repeats and CRISPR-associated protein 9 (CRISPR/Cas9). Pigs engineered to express human regulatory molecules such as CD46, CD55, and CD59, have produced promising results in preclinical models using nonhuman primates (NHPs) as recipients in combination with other human transgenes and costimulatory blockade-based immunosuppressive regimens [5–7]. Given this quantum leap forward, it is expected that clinical applications will soon commence. This review examines the role of the complement system in kidney xenotransplantation, including research updates and recent preclinical investigations.

## 2. Mechanism of hyperactivity of complement cascades in kidney xenograft

AMR, acute antibody-mediated rejection; AP, alternative pathway; ATG, anti-thymocyte immunoglobulin; C1-INH, C1 esterase inhibitor; CP, classical pathway; CR, complement receptor; CRISPR, clustered regulatory interspaced palindromic repeats; CRISPR/Cas9, CRISPR-associated protein 9; DAMP, damage-associated molecular pattern; DKO, double-xeno antigen knock-out; DSA, donor-specific antibody; GTKO, 1,3-galactosyltransferase knock out; HAR, hyper acute rejection; IRI, ischemia-reperfusion injury; IVIG, intravenous immunoglobulin; LP, lectin pathway; MAC, membrane attack complex; MASP, mannose-associated serine protease; MBL, mannose binding lectin; MGH, Massachusetts General Hospital; MMF, mycophenolate mofetil; NHP, nonhuman primate; PAMP, pathogen-associated molecular pattern; SLA, swine leukocyte antigen; TCMR, T-cell mediated rejection; TKO, triple-xeno antigen knock-out; TMA, thrombotic microangiopathy

The kidney is known to be particularly susceptible to complement activation owing to its glomerular structure, immune complex deposition in the mesangium and capillary wall, increased local complement protein production, and a lack of complement regulator expression [8–10]. The complement system is composed of more than 30 distinct serum- and membrane-associated proteins that are produced in the liver and circulate in the blood in an inactive form [11]. Complement activation occurs via three biochemical pathways: classical (antibody-dependent), alternative, and lectin, all of which contribute to both innate and adaptive immune responses [12,13]. When activated, the complement system generates a membrane attack complex (MAC), causing cell lysis, destruction of the graft vasculature, and ultimately graft failure. In xenotransplantation, the influencing factors are more likely to be activated, resulting in activation of the complement cascade, which is more difficult to control. Natural antibodies against three carbohydrate antigens, known as "xenoantigens," cause HAR in NHP recipients immediately after kidney xenograft perfusion [4]. Xenoantigens consist of three carbohydrate antigens found in all mammals except primates. They activate the complement cascade by binding to natural antibodies in humans, or NHPs. The interaction of preexisting antibodies against xenoantigenic epitopes on porcine endothelial cells triggers the complement cascade activation. This activation causes additional stimulation and lysis of endothelial cells, resulting in damage to the graft vasculature and graft failure [14]. Histologically, HAR is described as the loss of vascular integrity followed by edema, thrombosis, hemorrhage, and extensive vascular deposition of antibodies and terminal complement components [15].

### 2.1. Classical pathway

The classical pathway (CP) is initiated primarily by the binding of antibodies, such as IgM and IgG, to antigens. This binding causes the formation of the C1 complex, which consists of C1q and two molecules, C1r and C1s, resulting in the cleavage of C4 and C2 to form C4b2a (C3 convertase) (Figure 1) [11]. C4b2a cleaves C3 into C3a and C3b, while C3b combines with tC4b2a convertase to form C5 convertase, which splits C5 into C5a and C5b. C5b complexes with C6-9 to create the terminal membrane MAC, resulting in cell lysis and tissue damage. During xenotransplantation, the CP is activated when preformed antibodies in the recipient bind to xenoantigens present on the surface of the transplanted organ. Those antibodies are known as donor-specific antibodies (DSAs), specifically xeno-antibodies in xenotransplantation. This pathway contributes significantly to antibody-mediated rejection (AMR), including HAR. AMR occurs when the recipient's immune system attacks the transplanted organ after recognizing xenoantigens. The binding of DSAs to these antigens activates the CP, initiating a rapid immune response that causes vascular injury, thrombosis, and ultimately graft failure. Swine leukocyte antigens (SLA) can be targets of antibodies secreted by recipients. Other antigens, such as xenograft-derived neoantigens and damage-associated molecular patterns (DAMPs), are generated by ischemia reperfusion injury (IRI) [16]. These neo-antigens are also generated during infection, and attach to preformed IgM antibodies, which activate the following cascade. The CP not only causes direct damage through MAC formation but also acts indirectly by enhancing inflammation and recruiting other immune cells (neutrophils, monocytes, dendritic cells, and T and B cell) to the site of the graft, as the details are described below.

**2.2. Lectin pathway**

The lectin pathway (LP) is initiated by the binding of mannose-binding lectin (MBL) or ficolin, which is associated with MBL-associated serine protease 1 (MASP1), MASP2, and MASP3, to a pathogen-associated molecular pattern (PAMP) on the surface of an invading microorganism such as bacteria[17]. Furthermore, it can be activated by detecting DAMPs in damaged cells or transplanted tissues [18]. MASPs can cleave C4 and C2, similarly to C1s, and the same C3 convertase (C4bC2a) is produced (Figure 1) [17]. In xenotransplantation, MBL can detect neo-antigens expressed on the surface of pig following IRI, triggering the LP and the formation of inflammatory mediators (C3a and C5a) and the MAC [19]. This process contributes to xenograft rejection by causing cell lysis, inflammation, and immune cells recruitment, all of which can damage the transplanted organ. The LP functions alongside the CP and the alternative pathways (AP). Although antibody-antigen complexes generally initiate the CP and contributes significantly to HAR in xenotransplantation, the LP also plays an important role in complement activation through inflammation induced by infection, or IRI [16]. It can act to amplify immune responses either independently or in conjunction with the CP.

### 2.3. Alternative pathway

The AP is unique in that they can activate spontaneously without a target recognition molecule [20]. This pathway remains active at a low level due to the continuous hydrolysis of C3, a process known as "tick-over."[21] Activation can escalate rapidly when it encounters non-self surfaces, such as those presented by xenografts, particularly pig organs in primate recipients. When activated, the AP produces the C3 convertase (C3bBb), resulting in the MAC formation and cell lysis (Figure 1). Because the tick-over process occurs spontaneously, complement regulatory proteins play an important role in modulating the

AP activation. Notably, C3b produced by the CP or LP can also contribute to this process. Even when the CP initiates complement activation, the amplification loop of the AP may generate the majority of the downstream fragments [22]. Studies have demonstrated that IRI can trigger the AP through releasing of complement components, particularly C3, from renal tubular epithelium. Thereby, it is suggested the AP can be likely amplified on xenograft surfaces due to less compatibility of complement regulatory proteins and porcine organ vulnerability to IRI [23–26].

### 2.4. The complement roles in innate and adaptive immunity

All three pathways lead to the formation of a C3 convertase, which cleaves C3 into the split products C3a and C3b in the common pathway, resulting in C5 convertase cleaving C5 into C5a and C5b (Figure 1). The complement fragments such as C3a and C5a, collectively known as anaphylatoxins, mediate inflammatory responses by increasing vascular permeability, vasodilation, and the recruitment of immune cells such as neutrophils and macrophages [27,28]. The complement receptors C3aR, C5aR1, and C5aR2 are expressed on a variety of immune cells, including neutrophils, monocytes, dendritic cells, and T and B cells, where they help modulate immune cell chemotaxis and activation (Figure 1) [29–32]. The interactions of C3aR and C5aR with immune cells have direct implications in transplant rejection and inflammatory diseases [33,34]. Furthermore, complement activation can occur at specific sites within transplanted organs, such as the tubulointerstitium or peritubular capillaries [35,36]. In xenotransplantation, DAMPs and PAMPs are released from xenograft and subsequently phagocytosed by primate antigen-presenting cells such as dendritic cells and macrophages. This process transmits information to adaptive immune

cells independently of inflammation, often in conjunction with complement-mediated opsonization[37].

## 3. Specific responses causing or caused by complement activity in kidney xenotransplantation

Given the action of mechanisms, the specific responses that can cause or be caused by complement activity in kidney xenotransplantation are described below.

### 3.1. Antibody-mediated rejection

The complement system is pivotal role in AMR in kidney xenotransplantation. AMR is primarily driven by the CP of complement activation, which results in anaphylatoxin release and vascular cell lysis through MAC formation of the xenograft (Figure 1). AMR is significantly more likely to occur in xenotransplantation than in allotransplantation, because humans and NHPs naturally acquired DSAs against xenoantigens. In earlier experiments using preclinical models without genetically modified donor pigs, the natural antibodies caused AMR immediately after perfusion, resulting in HAR and graft loss during surgery [3]. Even if a recipient does not develop HAR, AMR is more likely to happen despite the use of heavier immunosuppressants. Antigen-antibody binding forms immune complexes that activate the complement cascade, culminating in the cleavage of C4 and the deposition of C4d on endothelial surfaces. The presence of C4d in peritubular capillaries is a well-established marker of complement activation and is routinely used in the diagnosis of AMR [38–40].

### 3.2. Thrombotic microangiopathy

Thrombotic microangiopathy (TMA) is an example of endothelial injury characterized by the formation of microthrombi in various blood vessels such as arteries, arterioles,

venules, and glomerular capillaries. This process is distinguished by the detachment of endothelial cells from the basement membrane, which results in the vascular exposure of thrombogenic surfaces and the activation of coagulation cascades. TMA can be triggered by AMR in both xeno- and allograft kidney transplantation (Figure 1) [41,42]. The deposition of complement proteins such as C4d and the presence of DSA is one of findings demonstrating AMR[43]. AMR from TMA can be difficult to be distinguish due to overlapping histological features, such as microthrombi consisting of fibrin and platelets. They can also arise independently, resulting in kidney graft loss [41,44–47], particularly in kidney xenotransplantation [48]. TMA also can be triggered by immunosuppressant drugs, infection, and IRI [41]. Furthermore, xenografts are more prone to cause TMA because of less compatibility of complement regulatory proteins expressed on porcine vascular cells. Additionally, coagulation dysregulation can cause TMA in kidney xenotransplantation [42,49]. The expression of multiple human transgenes of complement regulatory proteins and coagulation-related proteins in donor pig organs may help prevent TMA and AMR in xenotransplantation by mitigating complement-mediated endothelial damage and lowering the incidence of TMA [6]. Furthermore, regulating coagulation incompatibility has the potential to lower the risk and severity of TMA by expressing coagulation regulatory proteins on porcine endothelial cells [50,51].

### 3.3. T-cell mediated rejection

Although T-cell mediated rejection (TCMR) is not directly associated with complement systems such as AMR, there are significant links [13,52–54]. C3 is markedly upregulated in the tubules of kidney allografts and is deposited on the tubule surface adjacent to the T-cell infiltration [55]. Complement receptors, such as C3aR and C5aR, found on various immune cells, have also been linked to TCMR (Figure 1). Increased C5aR

expression on infiltrating cells during acute cellular rejection is associated with higher monocyte/macrophage infiltration and shorter graft survival [33]. Furthermore, C3aR on dendritic cells attenuates T-cell stimulation, whereas C3aR/C5aR activation weakens regulatory T-cell suppression, promoting pro-inflammatory responses [34,56] . The different C3 allotypes produced by the allograft may influence rejection, with donor-recipient complement mismatches influencing the severity [57,58]. Although C3 polymorphisms between pigs and primates have not been investigated, locally generated C3 in xenografts may be important in TCMR, influencing graft survival. Targeting complement receptors or regulating complement mismatches in donor pigs using human transgenes could improve xenotransplant outcomes.

### 3.4. Ischemia reperfusion injury

IRI can trigger complement activation through the release of endogenous ligands from necrotic and apoptotic cells, which activate the complement system via all three pathways (Figure 1) [59] . This activation leads to the formation of terminal complement components like C3a, C5a, and MAC, which amplify the inflammatory response and cause direct tubular cell injury [60]. IRI induces secretion of DAMPs from xenograft or surface expression of neoantigens on xenograft endothelial cells, leading to activate the complement cascade by binding to natural IgM antibodies and producing the antigen-antibody complex (Figure 1) [16,18,61,62]. Similarly, MBL binds to neoantigens, causing complement-mediated injury [63,64]. Activation of the complement system is crucial in the progression of kidney IRI. Research has demonstrated that the AP is also involved, as it can be triggered without the need for immunoglobulins [35,54,65,66]. Studies have shown that the renal allograft is a significant source of complement components such as C3, which is

upregulated during ischemia/reperfusion and contributes to inflammatory processes [25,65]. Conversely, the complement system can exacerbate IRI by promoting inflammation. The anaphylatoxins C3a and C5a, products of complement activation, interact with receptors on immune and non-immune cells, promoting leukocyte activation, chemotaxis, and increased vascular permeability, which contribute to tissue inflammation and injury [60]. C5aR and C3aR signaling have been shown to have deleterious effects[67,68]. An exposure of the porcine endothelial cells to human and NHP blood impairs production of extracellular cryoprotective, antithrombotic, and immunosuppressive adenosine, increasing the risk of IRI [69]. Porcine organ susceptibility to IRI may be attributable to this or an unknown feature of pig physiology [26].

### 3.5. Infection

Both bacterial and viral infections can cause vigorous complement activation, which poses a significant threat to xenograft survival owing to species incompatibility. During bacterial infections, the complement system identifies pathogens using two key mechanisms: the LP and the CP (Figure 1) [70]. Complement activation in the CP begins when C1 binds to antibodies on the bacterial surface, leading to MAC assembly. Similarly, bacterial cells are recognized by soluble-pattern recognition in the LP [70]. Similarly, viral infections can activate the complement system, particularly through the LP [71]. This promotes the deposition of C4 activation fragments in infected cells, which contributes to complement-mediated inflammation [72]. The CP also plays a role in viral infections, where immune complexes formed by viral antigens and antibodies activate C1, triggering complement activation. Cytomegalovirus, especially, porcine-derived cytomegalovirus can be transmitted to primate recipients [73]. The virus can activate the CP by promoting C1q binding to infected cells, resulting in the formation of the terminal complement complex

C5b-9, which ultimately causes cell lysis and death [74]. Due to need of heavy immunosuppressive regimen including anti-CD154/CD40 blockades to regulate rejection including HAR, xenotransplant recipients are usually susceptible to bacterial or viral infection[75]. To control infection is one of keys for xenotransplant recipients from the aspects of prevention of complement-mediated rejection.

## 4. Inhibitory strategies of complement activity in xenografts

Complement activity is crucial and causes significant complement-mediated damage in xenografts through the numerous mechanisms mentioned above. Moreover, in the context of xenotransplantation, it is indicated that complement regulatory proteins expressed on donor endothelial cells are far less compatible with complement system in recipients [76,77]. Several attempts have been made to overcome these concerns. In addition to gene-modification in donor pigs, some complement-targeting drugs approved by the Food and Drug Administration have been attempted in kidney xenotransplantation (Table 1).

### 4. 1. Complement regulatory proteins

Donors with xenoantigen knockouts have been used to inhibit complement activation and establish stable long-term survival as a result of gene engineering using CRISPR/Cas9 technology. Multiple human transgenes, including complement regulatory proteins, coagulation regulatory proteins, and inflammation/innate immune regulatory proteins, have also been inserted into donor pigs using gene editing. Along with development of immunosuppressive medications, these gene modifications have prolonged survival days in kidney xenotransplant NHPs (Figure 2) [37].

Transgenes encoding human complement regulatory proteins in pigs that have shown encouraging results in preclinical models include CD55 (decay-accelerating factor), CD46 (membrane cofactor protein), and CD59 (membrane inhibitor of reactive lysis). CD55 inhibits complement activation by promoting the decay of C3 and C5 convertases (Figure 1) [78,79]. Human CD55 transgenic pigs served as the vanguard for the successfully extending kidney xenograft survival in an NHP model (Figure 2) [80–82]. CD46 is a membrane-bound regulators that disrupts C3 convertase assembly by C3b inactivation [83,84]. Porcine kidneys with the human CD46 transgene into non-immunosuppressed baboons survived significantly longer than normal pig kidneys, with only minor macroscopic damage and focal tubular infarctions with viable renal tissue elsewhere (Figure 2) [85]. CD59 inhibits the formation of C5b-9 and MAC in cell membranes [86]. When kidneys from transgenic pigs expressing human CD59 and human CD55 were used, NHP recipients survived longer in the absence of immunosuppression than wild-type kidneys, indicating protection against HAR (Figure 2) [87]. Although these human complement regulatory transgenes reduced complement activity and alleviated HAR in preclinical models, the modifications were unable to counteract the hyperactivity of the complement system. To obtain long-term results, a combination of several additional transgenes and drugs must be used in addition to the human complement regulatory transgenes.

### 4.2. Complement inhibitory agents

#### 4.2.1. C5 inhibitors

Therapeutic strategies that target the complement pathways have shown the potential to manage AMR. C5 inhibitors, particularly eculizumab, have shown promise in allotransplantation for decreasing complement-mediated immune responses, such as AMR

[88–91]. Eculizumab is a humanized monoclonal antibody that binds to the C5 complement protein, inhibiting the terminal complement pathway, and blocks the formation of C5a (Figure 1). This potent anaphylatoxin triggers neutrophil recruitment and inflammation. Complement inhibition has emerged as an important method for protecting xenografts from immune-mediated injury in xenotransplantation. In brain-dead model, Locke et al. observed that xenograft biopsies did not show TMA or MAC deposition in the cases treated with eculizumab, although the appropriate interpretation of complement activity is difficult in a brain-dead model in which the complement system is usually activated [92]. In a study using porcine kidneys with double-xeno antigen knock-out (DKO), tesidolumab, another anti-C5 inhibitor, was used to block C5 activity and effective in preventing early acute AMR [93] . However, further research is needed to clearly define their roles in xenotransplantation.

**4.2.2. C1 esterase inhibitors**

Other inhibitors targeting upstream components of the complement cascade, such as C1 esterase inhibitors (C1-INH), are being investigated, with preliminary evidence indicating their potential benefits in preventing and treating AMR. Administration of C1-INH in xenotransplantation models has shown efficacy to prevent complement deposition and tissue damage [94–97]. This approach is particularly useful for blocking HAR and AMR through the CP, which is a significant challenge in xenograft rejection. However, Le Bas-Bernardet, et al. found IgM, C3, and C5b deposition in rejected xenograft without C4d staining, indicating that C1-INH blocked the CP but activated the AP (Figure 2) [98]. The results showed that regulating the AP and LP is also crucial for achieving long-term xenograft survival as well as regulating the CP.

**4.2.3. C3 inhibitors**

More clinically approved medicines may affect kidney xenotransplantation. Narsoplimab, a human monoclonal antibody targeting MASP-2, is a clinically approved LP inhibitor [99,100]. Pegcetacoplan, which blocks the AP by preventing C3 from generating C3b, has also been approved to treat IgA nephropathy and paroxysmal nocturnal hemoglobinuria [101]. In a human brain-dead decedent as a model of kidney xenotransplantation research, the agent was administered for AMR treatment along with steroids and plasmapheresis. The treatment successfully showed preservation of xenograft function [102]. Pegcetacoplan was also administered in the first clinical case as anti-rejection therapy due to the C3 deposition in xenograft biopsy specimen, and serum creatinine level was successfully recovered [103]. Cp40, another anti-C3 antibody, showed prevention of AMR and prolongation of graft survival in a highly sensitized NHP allotransplant model [104]. Moreover, cobra venom factor, depleting circulating C3, effectively suppressed AMR in NHP kidney allo- or xeno-transplant models (Figure 2) [105,106]. Although it requires further investigation in clinical use, it is indicated that inhibition of C3 is a potential strategy to prolong xenograft survivals by inhibition of complement mediated rejection or AMR.

**4.2.4. Intravenous immunoglobulin**

Intravenous immunoglobulin (IVIg) infusion provides various advantages for the prevention and/or treatment of AMR in sensitized kidney allo-transplant recipients [107–109]. IVIg suppresses both innate and adaptive immunity by reducing complement-mediated inflammation by inhibition of C5b-C9 and the MAC, increasing IgG clearance by interfering with the recycling system, and inhibiting cytokines and IL-10 production [110,111]. IVIg improves survival length of pig kidneys perfused with human blood [112]. Nevertheless, caution is advised, as it has been observed that high-dose IVIg significantly reduces the

exposure to concomitantly administered anti-C5 monoclonal antibodies [113]. Moreover, Mohiuddin M, et al. reported that IVIG bound strongly to porcine endothelial cells, which were observed in different lots of the product [121]. Although no complement-dependent cytotoxicity was observed against porcine endothelium, their data suggested that endothelial damage caused by anti-pig antibodies in IVIG might be more important in xenotransplantation than in allotransplantation. Despite these findings, further research is required to properly determine the efficacy and disadvantage of these innovative drugs in preventing long-term rejection in human xenograft recipients.

## 5. Recent progression in preclinical models

To overcome complement dysregulation and other challenges in xenotransplantation, gene modification in donor pigs has been tested using knockout or knock-in of various genes as well as combinations of immunosuppressive drugs including costimulatory blockades. In addition to the necessity of removing carbohydrate xenoantigens, human transgenes encoding complement regulatory proteins have helped to achieve long-term kidney xenograft survival in NHP models. After achieving a 90-day survival with pig kidneys with αGal knock-out (GTKO) [105], inserting the human CD55 transgene into GTKO pigs resulted in consistent long-term survival over a year (Table 2 and Figure 2) [7]. The advancement of gene engineering technology permitted the simultaneous editing of multiple genes in porcine donors, such as CRISPR/Cas9. Iwase, et al. demonstrated successful long-term survival of up to 260 days using pig kidneys with six human transgenes, including CD55 and CD46, as well as GTKO [114]. Deletion of three carbohydrate xenoantigens (αGal, Neu5GC, and Sda) (TKO) is considered the most appropriate for humans due to the lowest levels of complement-dependent cytotoxicity tests and anti-porcine IgG

or IgM antibodies [115–117]. However, preclinical results in NHPs using kidney xenografts with TKO have been inconsistent, even when multiple human transgenes, including complement regulatory proteins are inserted [118,119]. Yamamoto T, et al. found that even with multiple human transgenes including CD46 and CD55, long-term kidney xenograft survival was not achieved in TKO pigs (Table 2 and Figure 2) [118]. Therefore, despite advancement of human transgenes including complement regulatory proteins, TKO as the donor condition encountered another obstacle in the preclinical studies. Then, Ma D, et al. at Massachusetts General Hospital (MGH) firstly reported long-term survival of over 300 days by using TKO pigs with multiple human transgenes. They demonstrated the relevance of complement regulatory proteins by comparing TKO pigs with high expression of these proteins (CD46, CD55, and CD59) to those with low expression (Table 2 and Figure 2) [6]. Of note, the study also showed the superiority of anti-CD154 monoclonal antibody over anti-CD40 monoclonal antibody in the suppression of kidney xenograft rejection. Although anti-CD154 antibody has not been clinically approved due to thromboembolic side effects, the MGH group subsequently reported Fc-modified anti-CD154 prevented kidney allograft rejection without thrombotic events in NHP model [120]. Furthermore, the MGH group successfully achieved kidney xenotransplantation survival for over two years, a recent accomplishment using TKO pigs with multiple human transgenes expressing CD46 and CD55 (Figure 3) [5]. The study showed that xenograft survival was significantly longer in recipients of kidneys from TKO pigs with human transgenes than from those without (Table 2 and Figure 2). Despite the encouraging results, three out of the 15 recipients lost the xenografts due to TMA without AMR, indicating that there is still potential for improvement in complement discordant management. Interestingly, they also found C4d deposition in glomeruli but not in peritubular capillaries, without any other signs of AMR, even in the

long-term survivors. These findings suggested that C4d deposition may not be related to typical AMR in xenotransplantation, as C4 activation occurs prior to the intervention by complement regulatory proteins, as shown in Figure 1. The significance of C4d staining in the xenografts has yet to be determined.

Some studies have demonstrated the efficacy of complement inhibitors, such as C1-INH and anti-C5 antibodies. Adams et al. investigated the efficacy of the anti-C5 antibody, tesidolumab, which was found to reduce early graft loss due to AMR and improved graft survival in pig-to-rhesus kidney xenotransplantation models when compared to control recipients who did not receive the anti-C5 therapy (Table 2 and Figure 2) [93]. Although the kidneys of both groups showed significant IgM binding and C4d deposition, tesidolumab-treated kidneys had much less early C5b-9 staining than control kidneys. Eisenson et al. recently reported the successful long-term survival of kidneys from TKO pigs with multiple human transgenes, including CD46 and CD55, in baboons treated with C1-INH, anti-CD40 antibody, and anti-IL 6 antibody (Table 2 and Figure 2) [121]. Although consistent long-term survival was achieved for up to 337 days, it is unclear whether C1-INH is unavoidable for the protocol regimen, as no comparative studies regarding C1-INH have been reported in preclinical models. Further research is needed to determine the necessity for C1-INH and other complement regulatory agents.

## 6. Clinical models

Preclinical models have demonstrated successful long-term kidney xenograft survival using a combination of new drugs and donor pigs gene modifications. However, not all facilities have achieved long-term survival using clinical-grade TKO kidneys. Because pig gene modification is optimized for human, not NHPs, clinical xenotransplantation is

predicted to produce superior results. Despite advances in preclinical models, the clinical use of kidney xenotransplantation has yet to be approved. Some attempts have been made to use brain-dead models for short-term observations, demonstrating that HAR can be overcome using genetic engineering and drug development [122–125]. These investigations revealed a fresh insight: gene-edited pig organs function in the human body without HAR for a short period, but it is difficult to evaluate complement activity in brain-dead states, which are associated with immune and inflammatory responses, including complement activity. As a result, the data obtained from the decedent model is complicated, interpretation is difficult, and its clinical value is restricted. Four cases of clinical kidney xenotransplantation were reported at two distinct facilities through the compassionate approval by the Food and Drug Administration. In the first case performed by MGH, a C5 inhibitor (ravulizumab) was used as maintenance immunosuppression, and a C3 inhibitor (pegcetacoplan) was administered for anti-rejection therapy since C3 deposition was appreciated in the for-cause biopsy sample [103] . Two recipients at MGH received a kidney from exactly the same clone as those that demonstrated 2-year survival in NHPs. Unfortunately, the first recipient died from cardiovascular disease; however, the pathology findings of the xenograft revealed no evidence of rejection. These findings imply that complement-regulating strategies using gene modifications and novel agents are also effective to some extent.

## 7. Conclusion

The complement system plays an essential role in kidney xenotransplantation, and the mechanisms of complement activity have been studied. In addition to HAR and AMR, the complement cascade can activate a variety of immune responses, including TCMR, IRI,

and infection, resulting in xenograft failure. Novel drugs and gene modifications in donor pigs have been developed to modulate complement activity, allowing for the treatment of complement dysregulation by interfering with the three pathways. Preclinical investigations have revealed that complement regulation is critical for long-term survival in kidney xenotransplantation. The NHP model also suggests that complement dysregulation contributes to xenograft injury, necessitating a comprehensive strategy to address other impediments, such as xeno-antibodies, coagulation dysregulation and inflammatory activity. Future clinical applications will require a better understanding of the mechanisms and regulation of the complement system.

## Reference


1. Cooper DKC, Hara H, Iwase H, et al. Clinical Pig Kidney Xenotransplantation: How Close Are We? *J Am Soc Nephrol*. 2020;31(1):12-21. doi:10.1681/asn.2019070651

2. Goerlich CE, Kamberi SS, Ladowski J, et al. The Young Investigator Committee of the International Xenotransplantation Association—Perspective of advancements in the field in 2023. *Xenotransplantation*. 2024;31(1):e12845. doi:10.1111/xen.12845

3. Cooper DKC, Satyananda V, Ekser B, et al. Progress in pig-to-non-human primate transplantation models (1998–2013): a comprehensive review of the literature. *Xenotransplantation*. 2014;21(5):397-419. doi:10.1111/xen.12127

4. Cooper DKC, Ezzelarab MB, Hara H, et al. The pathobiology of pig-to-primate xenotransplantation: a historical review. *Xenotransplantation*. 2016;23(2):83-105. doi:10.1111/xen.12219

5. Anand RP, Layer JV, Heja D, et al. Design and testing of a humanized porcine donor for xenotransplantation. *Nature*. 2023;622(7982):393-401. doi:10.1038/s41586-023-06594-4

6. Ma D, Hirose T, Lassiter G, et al. Kidney transplantation from triple-knockout pigs expressing multiple human proteins in cynomolgus macaques. *Am J Transplant*. 2022;22(1):46-57. doi:10.1111/ajt.16780

7. Kim SC, Mathews DV, Breeden CP, et al. Long-term survival of pig-to-rhesus macaque renal xenografts is dependent on CD4 T cell depletion. *American Journal of Transplantation*. 2019;19(8):2174-2185. doi:10.1111/ajt.15329

8. Thurman JM, Nester CM. All Things Complement. *Clin J Am Soc Nephrol*. 2016;11(10):1856-1866. doi:10.2215/cjn.01710216

9. Thurman JM. Complement and the Kidney: An Overview. *Adv Chronic Kidney Dis*. 2020;27(2):86-94. doi:10.1053/j.ackd.2019.10.003

10. Petr V, Thurman JM. The role of complement in kidney disease. *Nat Rev Nephrol*. Published online 2023:1-17. doi:10.1038/s41581-023-00766-1

11. Walport MJ. Complement. *N Engl J Med*. 2001;344(14):1058-1066. doi:10.1056/nejm200104053441406

12. Java A, Atkinson J, Salmon J. Defective Complement Inhibitory Function Predisposes to Renal Disease. *Medicine*. 2013;64(1):307-324. doi:10.1146/annurev-med-072211-110606

13. Sacks SH, Chowdhury P, Zhou W. Role of the complement system in rejection. *Curr Opin Immunol*. 2003;15(5):487-492. doi:10.1016/s0952-7915(03)00100-6

14. Yang YG, Sykes M. Xenotransplantation: current status and a perspective on the future. *Nat Rev Immunol*. 2007;7(7):519-531. doi:10.1038/nri2099

15. Schuurman HJ, Pino-Chavez G, Phillips MJ, Thomas L, White DJG, Cozzi E. Incidence of hyperacute rejection in pig-to-primate transplantation using organs from hDAF-transgenic donors. *Transplantation*. 2002;73(7):1146-1151. doi:10.1097/00007890-200204150-00024

16. Horwitz JK, Chun NH, Heeger PS. Complement and Transplantation From New Mechanisms to Potential Biomarkers and Novel Treatment Strategies. *Clin Lab Med*. 2019;39(1):31-43. doi:10.1016/j.cll.2018.10.004

17. Fujita T. Evolution of the lectin–complement pathway and its role in innate immunity. *Nat Rev Immunol*. 2002;2(5):346-353. doi:10.1038/nri800

18. Land WG, Agostinis P, Gasser S, Garg AD, Linkermann A. DAMP—Induced Allograft and Tumor Rejection: The Circle Is Closing. *Am J Transplant*. 2016;16(12):3322-3337. doi:10.1111/ajt.14012

19. Bongoni AK, Kiermeir D, Jenni H, et al. Activation of the Lectin Pathway of Complement in Pig-to-Human Xenotransplantation Models. *Transplant J*. 2013;96(9):791-799. doi:10.1097/tp.0b013e3182a3a52b

20. Ehrnthaller C, Ignatius A, Gebhard F, Huber-Lang M. New Insights of an Old Defense System: Structure, Function, and Clinical Relevance of the Complement System. *Mol Med*. 2011;17(3-4):317-329. doi:10.2119/molmed.2010.00149

21. Lachmann PJ. The Amplification Loop of the Complement Pathways. *Adv Immunol*. 2009;104:115-149. doi:10.1016/s0065-2776(08)04004-2

22. Harboe M, Ulvund G, Vien L, Fung M, Mollnes TE. The quantitative role of alternative pathway amplification in classical pathway induced terminal complement activation. *Clin Exp Immunol*. 2004;138(3):439-446. doi:10.1111/j.1365-2249.2004.02627.x

23. Margarita R, Atousa A, R AW, J. PM, J.F. d'Apice A. Involvement of Both the Classical and Alternate Pathways of Complement in an Ex Vivo Model Of Xenograft Rejection. *Transplantation*. 1997;63(7):1021-1025. doi:10.1097/00007890-199704150-00020

24. Farrar CA, Zhou W, Lin T, Sacks SH. Local extravascular pool of C3 is a determinant of postischemic acute renal failure. *FASEB J*. 2006;20(2):217-226. doi:10.1096/fj.05-4747com

25. Thurman JM, Ljubanović D, Royer PA, et al. Altered renal tubular expression of the complement inhibitor Crry permits complement activation after ischemia/reperfusion. *J Clin Investig*. 2006;116(2):357-368. doi:10.1172/jci24521

26. Patel PM, Connolly MR, Coe TM, et al. Minimizing Ischemia Reperfusion Injury in Xenotransplantation. *Front Immunol*. 2021;12:681504. doi:10.3389/fimmu.2021.681504

27. Markiewski MM, Lambris JD. The Role of Complement in Inflammatory Diseases From Behind the Scenes into the Spotlight. *Am J Pathol*. 2007;171(3):715-727. doi:10.2353/ajpath.2007.070166

28. Dunkelberger JR, Song WC. Complement and its role in innate and adaptive immune responses. *Cell Res*. 2010;20(1):34-50. doi:10.1038/cr.2009.139

29. Daffern PJ, Pfeifer PH, Ember JA, Hugli TE. C3a is a chemotaxin for human eosinophils but not for neutrophils. I. C3a stimulation of neutrophils is secondary to eosinophil activation. *J Exp Med*. 1995;181(6):2119-2127. doi:10.1084/jem.181.6.2119

30. Hartmann K, Henz BM, Krüger-Krasagakes S, et al. C3a and C5a Stimulate Chemotaxis of Human Mast Cells. *Blood*. 1997;89(8):2863-2870. doi:10.1182/blood.v89.8.2863

31. Zwirner J, Werfel T, Wilken H, Theile E, Götze O. Anaphylatoxin C3a but not C3a(desArg) is a chemotaxin for the mouse macrophage cell line J774. *Eur J Immunol*. 1998;28(5):1570-1577. doi:10.1002/(sici)1521-4141(199805)28:05<1570::aid-immu1570>3.0.co;2-6

32. Vandendriessche S, Cambier S, Proost P, Marques PE. Complement Receptors and Their Role in Leukocyte Recruitment and Phagocytosis. *Front Cell Dev Biol*. 2021;9:624025. doi:10.3389/fcell.2021.624025

33. Gueler F, Rong S, Gwinner W, et al. Complement 5a Receptor Inhibition Improves Renal Allograft Survival. *J Am Soc Nephrol*. 2008;19(12):2302-2312. doi:10.1681/asn.2007111267

34. Li K, Anderson KJ, Peng Q, et al. Cyclic AMP plays a critical role in C3a-receptor–mediated regulation of dendritic cells in antigen uptake and T-cell stimulation. *Blood*. 2008;112(13):5084-5094. doi:10.1182/blood-2008-05-156646

35. Thurman JM, Ljubanovic D, Edelstein CL, Gilkeson GS, Holers VM. Lack of a Functional Alternative Complement Pathway Ameliorates Ischemic Acute Renal Failure in Mice. *J Immunol*. 2003;170(3):1517-1523. doi:10.4049/jimmunol.170.3.1517

36. Stites E, Quintrec ML, Thurman JM. The Complement System and Antibody-Mediated Transplant Rejection. *J Immunol*. 2015;195(12):5525-5531. doi:10.4049/jimmunol.1501686

37. Miyagawa S, Maeda A, Toyama C, et al. Aspects of the Complement System in New Era of Xenotransplantation. *Front Immunol*. 2022;13:860165. doi:10.3389/fimmu.2022.860165

38. Herzenberg AM, Gill JS, Djurdjev O, Magil AB. C4d Deposition in Acute Rejection: An Independent Long-Term Prognostic Factor. *J Am Soc Nephrol*. 2002;13(1):234-241. doi:10.1681/asn.v131234

39. Feucht HE, Schneeberger H, Hillebrand G, et al. Capillary deposition of C4d complement fragment and early renal graft loss. *Kidney Int*. 1993;43(6):1333-1338. doi:10.1038/ki.1993.187

40. Lederer SR, Kluth-Pepper B, Schneeberger H, Albert E, Land W, Feucht HE. Impact of humoral alloreactivity early after transplantation on the long-term survival of renal allografts. *Kidney Int*. 2001;59(1):334-341. doi:10.1046/j.1523-1755.2001.00495.x

41. Imanifard Z, Liguori L, Remuzzi G. TMA in Kidney Transplantation. *Transplantation*. 2023;107(11):2329-2340. doi:10.1097/tp.0000000000004585

42. Shimizu A, Yamada K, Robson SC, Sachs DH, Colvin RB. Pathologic Characteristics of Transplanted Kidney Xenografts. *J Am Soc Nephrol*. 2012;23(2):225-235. doi:10.1681/asn.2011040429

43. Naesens M, Roufosse C, Haas M, et al. The Banff 2022 Kidney Meeting Report: Reappraisal of microvascular inflammation and the role of biopsy-based transcript diagnostics. *Am J Transplant*. 2024;24(3):338-349. doi:10.1016/j.ajt.2023.10.016

44. Jodele S, Dandoy CE, Lane A, et al. Complement blockade for TA-TMA: lessons learned from a large pediatric cohort treated with eculizumab. *Blood*. 2020;135(13):1049-1057. doi:10.1182/blood.2019004218

45. Palma LMP, Sridharan M, Sethi S. Complement in Secondary Thrombotic Microangiopathy. *Kidney Int Rep*. 2021;6(1):11-23. doi:10.1016/j.ekir.2020.10.009

46. Sperati CJ. How I Treat Complement-Mediated TMA. *CJASN*. 2022;17(3):452-454. doi:10.2215/cjn.13581021

47. Genest DS, Patriquin CJ, Licht C, John R, Reich HN. Renal Thrombotic Microangiopathy: A Review. *Am J Kidney Dis*. 2023;81(5):591-605. doi:10.1053/j.ajkd.2022.10.014

48. Rosales IA, Colvin RB. The pathology of solid organ xenotransplantation. *Curr Opin Organ Transplant*. 2019;24(5):535-542. doi:10.1097/mot.0000000000000681

49. 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(5):1006-1016. doi:10.1111/j.1600-6143.2009.02602.x

50. Iwase H, Ekser B, Hara H, et al. Regulation of human platelet aggregation by genetically modified pig endothelial cells and thrombin inhibition. *Xenotransplantation*. 2014;21(1):72-83. doi:10.1111/xen.12073

51. Kim H, Hawthorne WJ, Kang HJ, et al. Human thrombomodulin regulates complement activation as well as the coagulation cascade in xeno-immune response. *Xenotransplantation*. 2015;22(4):260-272. doi:10.1111/xen.12173

52. Anwar IJ, DeLaura I, Ladowski J, Gao Q, Knechtle SJ, Kwun J. Complement-targeted therapies in kidney transplantation—insights from preclinical studies. *Front Immunol*. 2022;13:984090. doi:10.3389/fimmu.2022.984090

53. Cernoch M, Viklicky O. Complement in Kidney Transplantation. *Front Med*. 2017;4:66. doi:10.3389/fmed.2017.00066

54. Grafals M, Thurman JM. The Role of Complement in Organ Transplantation. *Front Immunol*. 2019;10:2380. doi:10.3389/fimmu.2019.02380

55. Lakkis FG. Transplant rejection: Mind your T-cell language. *Nat Med*. 2002;8(10):1043-1043. doi:10.1038/nm1002-1043a

56. Kwan W hong, Touw W van der, Paz-Artal E, Li MO, Heeger PS. Signaling through C5a receptor and C3a receptor diminishes function of murine natural regulatory T cells. *J Exp Med*. 2013;210(2):257-268. doi:10.1084/jem.20121525

57. Pratt JR, Basheer SA, Sacks SH. Local synthesis of complement component C3 regulates acute renal transplant rejection. *Nat Med*. 2002;8(6):582-587. doi:10.1038/nm0602-582

58. Tang S, Zhou W, Sheerin NS, Vaughan RW, Sacks SH. Contribution of renal secreted complement C3 to the circulating pool in humans. *J Immunol (Baltim, Md : 1950)*. 1999;162(7):4336-4341.

59. Arumugam TV, Shiels IA, Woodruff TM, Granger DN, Taylor SM. The Role of the Complement System in Ischemia-Reperfusion Injury. *Shock*. 2004;21(5):401-409. doi:10.1097/00024382-200405000-00002

60. Danobeitia JS, Djamali A, Fernandez LA. The role of complement in the pathogenesis of renal ischemia-reperfusion injury and fibrosis. *Fibrogenesis Tissue Repair*. 2014;7(1):16. doi:10.1186/1755-1536-7-16

61. Atkinson C, Qiao F, Yang X, et al. Targeting Pathogenic Postischemic Self-Recognition by Natural IgM to Protect Against Posttransplantation Cardiac Reperfusion Injury. *Circulation*. 2015;131(13):1171-1180. doi:10.1161/circulationaha.114.010482

62. Zhang M, Carroll MC. Natural IgM-mediated innate autoimmunity: a new target for early intervention of ischemia–reperfusion injury. *Expert Opin Biol Ther*. 2007;7(10):1575-1582. doi:10.1517/14712598.7.10.1575

63. Zhang M, Alicot EM, Chiu I, et al. Identification of the target self-antigens in reperfusion injury. *J Exp Med*. 2006;203(1):141-152. doi:10.1084/jem.20050390

64. Land WG, Agostinis P, Gasser S, Garg AD, Linkermann A. Transplantation and Damage-Associated Molecular Patterns (DAMPs). *Am J Transplant*. 2016;16(12):3338-3361. doi:10.1111/ajt.13963

65. Thurman JM, Lucia MS, Ljubanovic D, Holers VM. Acute tubular necrosis is characterized by activation of the alternative pathway of complement. *Kidney Int*. 2005;67(2):524-530. doi:10.1111/j.1523-1755.2005.67109.x

66. Thurman JM, Royer PA, Ljubanovic D, et al. Treatment with an Inhibitory Monoclonal Antibody to Mouse Factor B Protects Mice from Induction of Apoptosis and Renal Ischemia/Reperfusion Injury. *J Am Soc Nephrol*. 2006;17(3):707-715. doi:10.1681/asn.2005070698

67. Peng Q, Li K, Smyth LA, et al. C3a and C5a Promote Renal Ischemia-Reperfusion Injury. *J Am Soc Nephrol*. 2012;23(9):1474-1485. doi:10.1681/asn.2011111072

68. Vries B de, Köhl J, Leclercq WKG, et al. Complement Factor C5a Mediates Renal Ischemia-Reperfusion Injury Independent from Neutrophils. *J Immunol*. 2003;170(7):3883-3889. doi:10.4049/jimmunol.170.7.3883

69. Khalpey Z, Yuen AH, Kalsi KK, et al. Loss of ecto-5′nucleotidase from porcine endothelial cells after exposure to human blood: Implications for xenotransplantation. *Biochim Biophys Acta (BBA) - Mol Basis Dis*. 2005;1741(1-2):191-198. doi:10.1016/j.bbadis.2005.03.008

70. Heesterbeek DAC, Angelier ML, Harrison RA, Rooijakkers SHM. Complement and Bacterial Infections: From Molecular Mechanisms to Therapeutic Applications. *J Innate Immun*. 2018;10(5-6):455-464. doi:10.1159/000491439

71. Perico L, Benigni A, Casiraghi F, Ng LFP, Renia L, Remuzzi G. Immunity, endothelial injury and complement-induced coagulopathy in COVID-19. *Nat Rev Nephrol*. 2021;17(1):46-64. doi:10.1038/s41581-020-00357-4

72. Eddie-Ip WK, Chan KH, Law HKW, et al. Mannose-Binding Lectin in Severe Acute Respiratory Syndrome Coronavirus Infection. *J Infect Dis*. 2005;191(10):1697-1704. doi:10.1086/429631

73. Fishman JA. Risks of Infectious Disease in Xenotransplantation. *New Engl J Med*. 2022;387(24):2258-2267. doi:10.1056/nejmra2207462

74. Java A, Edwards A, Rossi A, et al. Cytomegalovirus-induced thrombotic microangiopathy after renal transplant successfully treated with eculizumab: case report and review of the literature. *Transpl Int*. 2015;28(9):1121-1125. doi:10.1111/tri.12582

75. Habibabady Z, McGrath G, Kinoshita K, et al. Antibody-mediated rejection in xenotransplantation: Can it be prevented or reversed? *Xenotransplantation*. 2023;30(4):e12816. doi:10.1111/xen.12816

76. Miyagawa S, Hirose H, Shirakura R, et al. THE MECHANISM OF DISCORDANT XENOGRAFT REJECTION. *Transplantation*. 1988;46(6):825-829. doi:10.1097/00007890-198812000-00007

77. Seya T, Mikata S, Fukui A, Murakami Y, Matsumoto M, Nagasawa S. Molecular remodeling of complement regulatory proteins for xenotransplantation. *Immunopharmacology*. 1999;42(1-3):75-80. doi:10.1016/s0162-3109(99)00016-8

78. Brodbeck WG, Kuttner-Kondo L, Mold C, Medof ME. Structure/function studies of human decay-accelerating factor. *Immunology*. 2000;101(1):104-111. doi:10.1046/j.1365-2567.2000.00086.x

79. Medof ME, Kinoshita T, Nussenzweig V. Inhibition of complement activation on the surface of cells after incorporation of decay-accelerating factor (DAF) into their membranes. *J Exp Med*. 1984;160(5):1558-1578. doi:10.1084/jem.160.5.1558

80. Zaidi A, Schmoeckel M, Bhatti F, et al. Life-Supporting Pig-To-Primate Renal Xenotransplantation Using Genetically Modified Donors. *Transplantation*. 1998;65(12):1584-1590. doi:10.1097/00007890-199806270-00008

81. 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(4):300-310. doi:10.1034/j.1399-3089.2003.02014.x

82. Cavicchioli L, Zan GD, Zappulli V, et al. Histopathological findings in the gastrointestinal tract of primate recipients of porcine renal xenografts following different immunosuppressive regimens. *Xenotransplantation*. 2007;14(2):145-156. doi:10.1111/j.1399-3089.2007.00382.x

83. Seya T, Turner JR, Atkinson JP. Purification and characterization of a membrane protein (gp45-70) that is a cofactor for cleavage of C3b and C4b. *J Exp Med*. 1986;163(4):837-855. doi:10.1084/jem.163.4.837

84. Liszewski MK, Leung M, Cui W, et al. Dissecting Sites Important for Complement Regulatory Activity in Membrane Cofactor Protein (MCP; CD46)*. *J Biol Chem*. 2000;275(48):37692-37701. doi:10.1074/jbc.m004650200

85. Loveland BE, Milland J, Kyriakou P, et al. Characterization of a CD46 transgenic pig and protection of transgenic kidneys against hyperacute rejection in non-immunosuppressed

baboons. *Xenotransplantation*. 2004;11(2):171-183. doi:10.1046/j.1399-3089.2003.00103_11_2.x

86. Huang Y, Qiao F, Abagyan R, Hazard S, Tomlinson S. Defining the CD59-C9 Binding Interaction*. *J Biol Chem*. 2006;281(37):27398-27404. doi:10.1074/jbc.m603690200

87. Ménoret S, Plat M, Blancho G, et al. Characterization of Human CD55 and CD59 Transgenic Pigs and Kidney Xenotransplantation in the Pig-to-Baboon Combination. *Transplantation*. 2004;77(9):1468-1471. doi:10.1097/01.tp.0000111758.35048.ea

88. Locke JE, Magro CM, Singer AL, et al. The Use of Antibody to Complement Protein C5 for Salvage Treatment of Severe Antibody-Mediated Rejection. *Am J Transplant*. 2009;9(1):231-235. doi:10.1111/j.1600-6143.2008.02451.x

89. Kanbay M, Copur S, Yilmaz ZY, et al. The role of anticomplement therapy in the management of the kidney allograft. *Clin Transplant*. 2024;38(3):e15277. doi:10.1111/ctr.15277

90. Tan EK, Bentall AJ, Dean PG, Shaheen MF, Stegall MD, Schinstock CA. Use of Eculizumab for Active Antibody-Mediated Rejection that Occurs Early Post-Kidney Transplantation. *Transplantation*. 2019;Publish Ahead of Print(NA;):NA; doi:10.1097/tp.0000000000002639

91. Chehade H, Rotman S, Matter M, Girardin E, Aubert V, Pascual M. Eculizumab to Treat Antibody-Mediated Rejection in a 7-Year-Old Kidney Transplant Recipient. *Pediatrics*. 2015;135(2):e551-e555. doi:10.1542/peds.2014-2275

92. Jones-Carr ME, Fatima H, Kumar V, et al. C5 inhibition with eculizumab prevents thrombotic microangiopathy in a case series of pig-to-human kidney xenotransplantation. *J Clin Investig*. 2024;134(5):e175996. doi:10.1172/jci175996

93. Adams AB, Lovasik BP, Faber DA, et al. Anti-C5 Antibody Tesidolumab Reduces Early Antibody-mediated Rejection and Prolongs Survival in Renal Xenotransplantation. *Ann Surg*. 2021;274(3):473-480. doi:10.1097/sla.0000000000004996

94. Hecker JM, Lorenz R, Appiah R, et al. C1-inhibitor for prophylaxis of xenograft rejection after pig to cynomolgus monkey kidney transplantation. *Transplantation*. 2002;73(5):688-694. doi:10.1097/00007890-200203150-00006

95. Brigitte HÖ, Annette W, Michael K. Protection of Porcine Endothelial Cells from Complement-Mediated Cytotoxicity by the Human Complement Regulators CD59, C1 Inhibitor, and Soluble Complement Receptor Type 1. *Transplantation*. 1996;62(11):1693-1696. doi:10.1097/00007890-199612150-00032

96. Vangerow B, Hecker JM, Lorenz R, et al. C1-Inhibitor for treatment of acute vascular xenograft rejection in cynomolgus recipients of h-DAF transgenic porcine kidneys. *Xenotransplantation*. 2001;8(4):266-272. doi:10.1034/j.1399-3089.2001.00130.x

97. Dalmasso AP, Plati JL. Prevention Of Complement-Mediated Activation Of Xenogeneic Endothelial Cells In An In Vitro Model Of Xenograft Hyperacute Rejection By C1 Inhibitor. *Transplantation*. 1993;56(5):1171-1176. doi:10.1097/00007890-199311000-00024

98. Bas-Bernardet SL, Tillou X, Branchereau J, et al. Bortezomib, C1-Inhibitor and Plasma Exchange Do Not Prolong the Survival of Multi-Transgenic GalT-KO Pig Kidney Xenografts in Baboons. *Am J Transplant*. 2015;15(2):358-370. doi:10.1111/ajt.12988

99. Pandrowala A, Ganatra P, Krishnan VP, et al. Narsoplimab for severe transplant-associated thrombotic microangiopathy. *Thromb J*. 2023;21(1):26. doi:10.1186/s12959-023-00464-9

100. Young JA, Pallas CR, Knovich MA. Transplant-associated thrombotic microangiopathy: theoretical considerations and a practical approach to an unrefined diagnosis. *Bone Marrow Transplant*. 2021;56(8):1805-1817. doi:10.1038/s41409-021-01283-0

101. Vivarelli M, Barratt J, Beck LH, et al. The Role of Complement in Kidney Disease: Conclusions From a Kidney Disease: Improving Global Outcomes (KDIGO) Controversies Conference. *Kidney Int*. Published online 2024. doi:10.1016/j.kint.2024.05.015

102. Mattoo A, Tatapudi V, Stern J, et al. V-312.1: Evaluation of proteinuria and microalbuminuria in a long-term pig-to-human xenothymokidney study. *Transplantation*. 2024;108(9S). doi:10.1097/01.tp.0001066876.60557.56

103. Kawai T, Williams WW, Elias N, et al. Xenotransplantation of a Porcine Kidney for End-Stage Kidney Disease. *N Engl J Med*. Published online February 6, 2025. doi:10.1056/nejmoa2412747

104. Schmitz R, Fitch ZW, Schroder PM, et al. C3 complement inhibition prevents antibody-mediated rejection and prolongs renal allograft survival in sensitized non-human primates. *Nat Commun*. 2021;12(1):5456. doi:10.1038/s41467-021-25745-7

105. Yamada K, Yazawa K, Shimizu A, et al. Marked prolongation of porcine renal xenograft survival in baboons through the use of α1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. *Nat Med*. 2005;11(1):32-34. doi:10.1038/nm1172

106. (Song) SC, Zhong S, Xiang Y, et al. Complement Inhibition Enables Renal Allograft Accommodation and Long-Term Engraftment in Presensitized Nonhuman Primates. *Am J Transplant*. 2011;11(10):2057-2066. doi:10.1111/j.1600-6143.2011.03646.x

107. Vo AA, Choi J, Cisneros K, et al. Benefits of Rituximab Combined With Intravenous Immunoglobulin for Desensitization in Kidney Transplant Recipients. *Transplantation*. 2014;98(3):312-319. doi:10.1097/tp.0000000000000064

108. Jordan SC, Vo A, Bunnapradist S, et al. Intravenous immune globulin treatment inhibits crossmatch positivity and allows for successful transplantation of incompatible organs in living-donor and cadaver recipients1. *Transplantation*. 2003;76(4):631-636. doi:10.1097/01.tp.0000080685.31697.fc

109. Kakuta Y, Satoh S, Watarai Y, et al. Successful Desensitization of T cell Flow Cytometry Crossmatch Positive Renal Transplant Recipients Using Plasmapheresis and Super High-Dose Intravenous Immunoglobulin. *Transplant Direct*. 2017;4(1):e336. doi:10.1097/txd.0000000000000753

110. Galeotti C, Kaveri SV, Bayry J. IVIG-mediated effector functions in autoimmune and inflammatory diseases. *Int Immunol*. 2017;29(11):491-498. doi:10.1093/intimm/dxx039

111. Tha-In T, Bayry J, Metselaar HJ, Kaveri SV, Kwekkeboom J. Modulation of the cellular immune system by intravenous immunoglobulin. *Trends Immunol*. 2008;29(12):608-615. doi:10.1016/j.it.2008.08.004

112. Fiane, Videm, Mellbye, et al. Immunoglobulin Prolongs Survival of Pig Kidneys Perfused Ex Vivo with Human Blood. *Scand J Immunol*. 1998;47(6):568-574. doi:10.1046/j.1365-3083.1998.00341.x

113. Jordan SC, Kucher K, Bagger M, et al. Intravenous immunoglobulin significantly reduces exposure of concomitantly administered anti-C5 monoclonal antibody tesidolumab. *Am J Transplant*. 2020;20(9):2581-2588. doi:10.1111/ajt.15922

114. 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):e12293. doi:10.1111/xen.12293

115. Gao B, Long C, Lee W, et al. Anti-Neu5Gc and anti-non-Neu5Gc antibodies in healthy humans. *Plos One*. 2017;12(7):e0180768. doi:10.1371/journal.pone.0180768

116. Cooper DKC, Hara H, Iwase H, et al. Justification of specific genetic modifications in pigs for clinical organ xenotransplantation. *Xenotransplantation*. 2019;26(4):e12516. doi:10.1111/xen.12516

117. Li Q, Shaikh S, Iwase H, et al. Carbohydrate antigen expression and anti-pig antibodies in New World capuchin monkeys: Relevance to studies of xenotransplantation. *Xenotransplantation*. 2019;26(3):e12498. doi:10.1111/xen.12498

118. Yamamoto T, Iwase H, Patel D, et al. Old World Monkeys are less than ideal transplantation models for testing pig organs lacking three carbohydrate antigens (Triple-Knockout). *Sci Rep-uk*. 2020;10(1):9771. doi:10.1038/s41598-020-66311-3

119. Ariyoshi Y, Takeuchi K, Pomposelli T, et al. Antibody reactivity with new antigens revealed in multi-transgenic triple knockout pigs may cause early loss of pig kidneys in baboons. *Xenotransplantation*. 2021;28(1):e12642. doi:10.1111/xen.12642

120. Lassiter G, Otsuka R, Hirose T, et al. TNX-1500, a crystallizable fragment–modified anti-CD154 antibody, prolongs nonhuman primate renal allograft survival. *Am J Transplant*. 2023;23(8):1171-1181. doi:10.1016/j.ajt.2023.03.022

121. Eisenson D, Hisadome Y, Santillan M, et al. Consistent survival in consecutive cases of life-supporting porcine kidney xenotransplantation using 10GE source pigs. *Nat Commun*. 2024;15(1):3361. doi:10.1038/s41467-024-47679-6

122. Locke JE, Kumar V, Anderson D, Porrett PM. Normal Graft Function After Pig-to-Human Kidney Xenotransplant. *JAMA Surg*. 2023;158(10):1106-1108. doi:10.1001/jamasurg.2023.2774

123. Montgomery RA, Stern JM, Lonze BE, et al. Results of Two Cases of Pig-to-Human Kidney Xenotransplantation. *New Engl J Med*. 2022;386(20):1889-1898. doi:10.1056/nejmoa2120238

124. Porrett PM, Orandi BJ, Kumar V, et al. First clinical-grade porcine kidney xenotransplant using a human decedent model. *Am J Transplant*. Published online 2022. doi:10.1111/ajt.16930

125. Loupy A, Goutaudier V, Giarraputo A, et al. Immune response after pig-to-human kidney xenotransplantation: a multimodal phenotyping study. *Lancet*. 2023;402(10408):1158-1169. doi:10.1016/s0140-6736(23)01349-1

Table 1. Complement inhibitors and regulators in kidney xenotransplantation

| Intervention | Target | Function |
|---|---|---|
| Drug | | |
| C1-INH | C1 esterase | Inhibits C1 esterase |
| MASP-2 inhibitor | MASP-2 | Inhibits MASP-2 forming complex |
| C3 inhibitor | C3 | Prevents C3 from generating C3b |
| C5 inhibitor | C5 | Prevents C5 cleavage |
| IVIg | C5b-C9 and MAC | Inhibits MAC formation |
| Human transgene in donor pigs | | |
| CD46 | C3 convertase | Cleaves C3b and C4b |
| CD55 | C3/C5 convertase | Promotes decay of C3 and C5 |
| CD59 | MAC | Prevents MAC formation |

## Table 1 caption

C1-INH, C1 esterase inhibitor; IVIg, intravenous immunoglobulin; MAC, membrane attack complex; MASP, mannose-associated serine protease.

Table 2. Recent outcomes of kidney xenotransplantation in NHP model by manipulating complement

| Author, year published | NHP species | KO xeno-antigen | Insertion of complement regulatory proteins | Insertion of other human transgenes and phenotypes | Complement inhibitor | Induction of immusuppressants | Maintenance immunosuppressants | The longest survival days (MST) |
|---|---|---|---|---|---|---|---|---|
| Kim CS, 2019 [7] | Rhesus | GTKO | **CD55** | - | - | aCD4, +/-aCD8 | aCD154, MMF, steroid | 499 (328) |
| Yamamoto T, 2020 [118] | Baboons | TKO | **CD46, CD55** | EPCR/TBM/CD47/HO1 | - | ATG, aCD20 | aCD40, Rapa, steroid | 61 (4) |
| Adams AB, 2021 [93] | Rhesus | DKO | - | ±SLA-1 KO | -<br>**C5 inhibitor** | aCD4, aCD20 | aCD154, MMF, steroid | 435 (9)<br>557 (205) |
| Ma D, 2021 [6] | Cynomolgus | TKO | CD46, CD55, CD59 (low expression)<br>**CD46, CD55, CD59 (high expression)** | HLA-E/CD47<br>HLA-E/CD47/PD-L1 | - | ATG, aCD20 | aCD154, Tac/Rapa, MMF, steroid | 61 (31.5)<br>316 (103) |
| Anand RP, 2023 [5] | Cynomolgus | TKO | -<br>**CD46, CD55** | ±PERV inactivation<br>EPCR/TBM/CD47/HO1/A20/±PERV | - | ATG, aCD20 | aCD154, Tac, MMF, steroid | 50 (23.5)<br>758 (176) |
| Eisenson D, 2023 [121] | Baboons | TKO | **CD46, CD55** | EPCR/TBM/CD47/HO1/GHR KO | **C1-INH** | ATG, aCD20 | aCD40, aIL-6R, MMF, sirolimus | 337 (278) |

## Table 2 Caption

aCD154, anti-CD154 antibody; aCD20, anti-CD20 antibody; aCD4, anti-CD4 antibody; aCD40, anti-CD40 antibody; aCD8, anti-CD8 antibody; ATG, anti-thymocyte globulin; DKO, double-knock out of xeno carbohydrate antigens; EPCR, endothelial protein C receptor; GHR, growth hormone receptor; GTKO, gal transferase knock out; MMF, mycophenolate mofetil; MST, median survival time; PERV, porcine endothelial retrovirus; Rapa, rapamaycin; Tac, tacrolimus; SLA, swine leukocyte antigen; TBM, thrombomodulin; TKO, triple-knock out of xeno carbohydrate antigens;

## Figure 1 Caption

Figure 1. The complement cascade activation and complement-inhibitory treatment in xenografts.

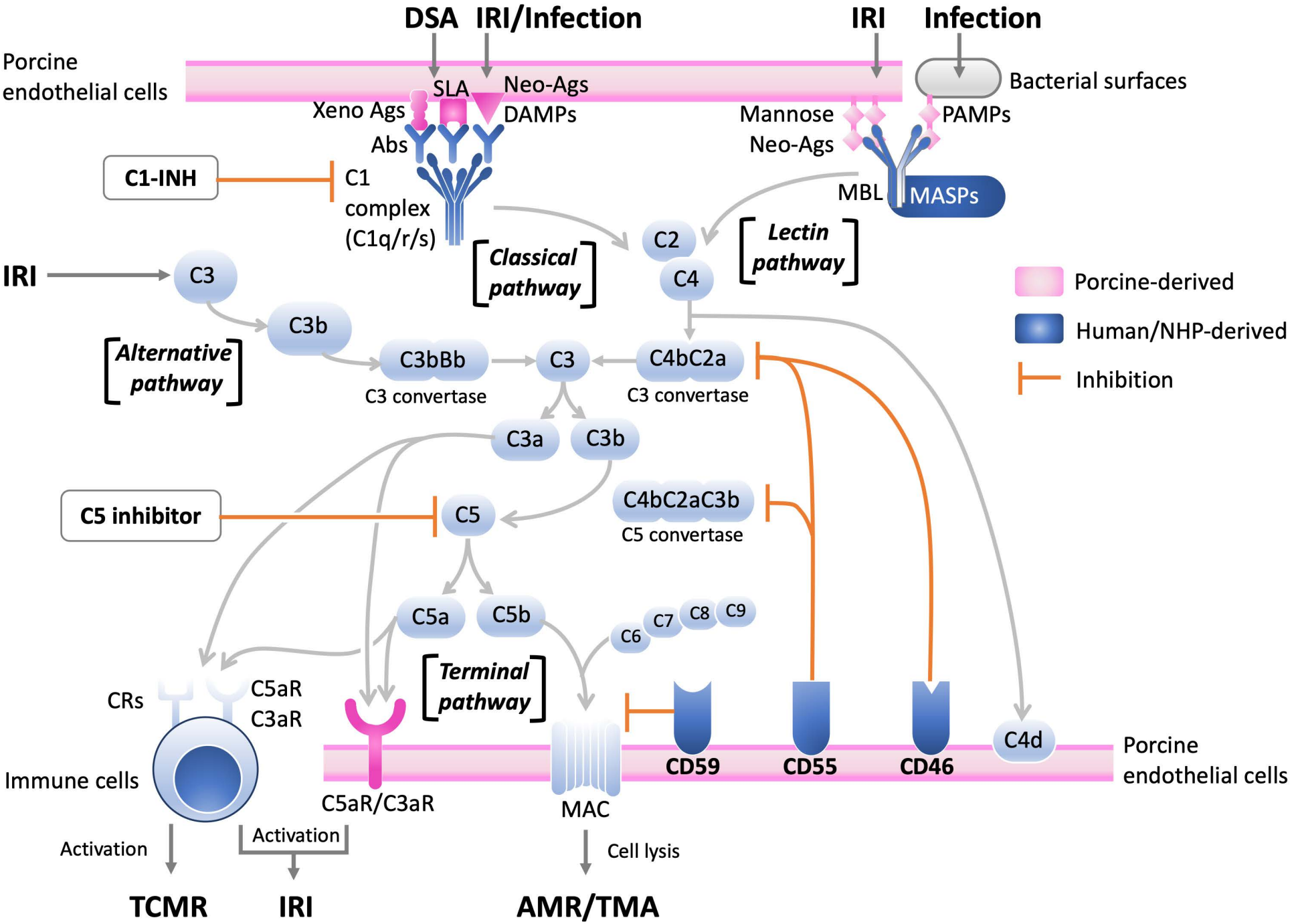
Ab, antibody; Ag, antigen; AMR, antibody-mediated rejection; C1-INH, C1 esterase inhibitor; C5aR, C5a receptor; C3aR, C3a receptor; CR, complement receptor; DAMP, damage-associated molecular pattern; DSA, donor-specific antibody; IRI, ischemia-reperfusion injury; MAC, membrane attack complex; MASP, mannose-associated serine protease; MBL, mannose binding lectin; NHP, nonhuman primate; PAMP, pathogen-associated molecular pattern; SLA, swine leukocyte antigen; TCMR, T-cell mediated rejection; TMA, thrombotic microangiopathy

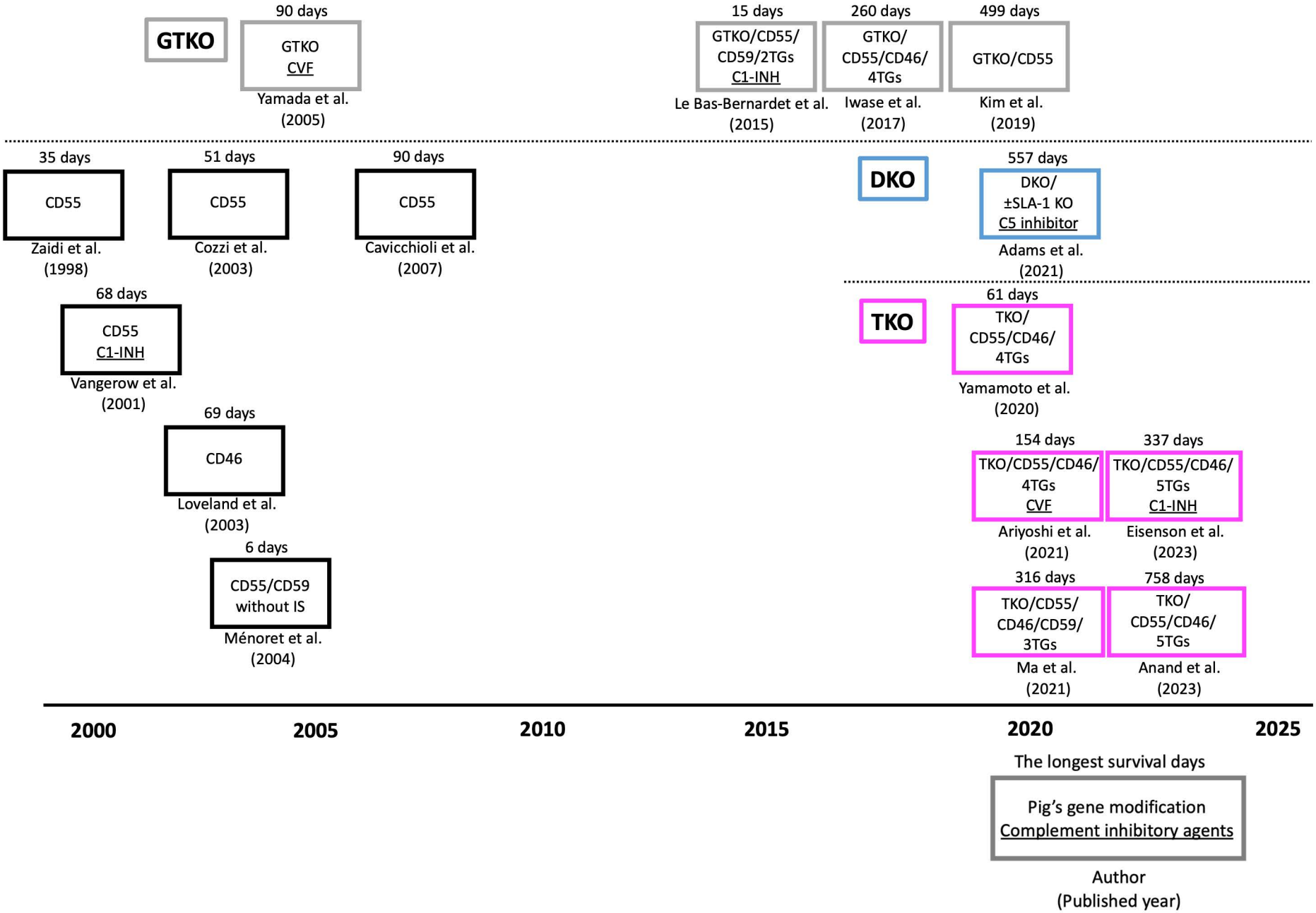
## Figure 2 Caption

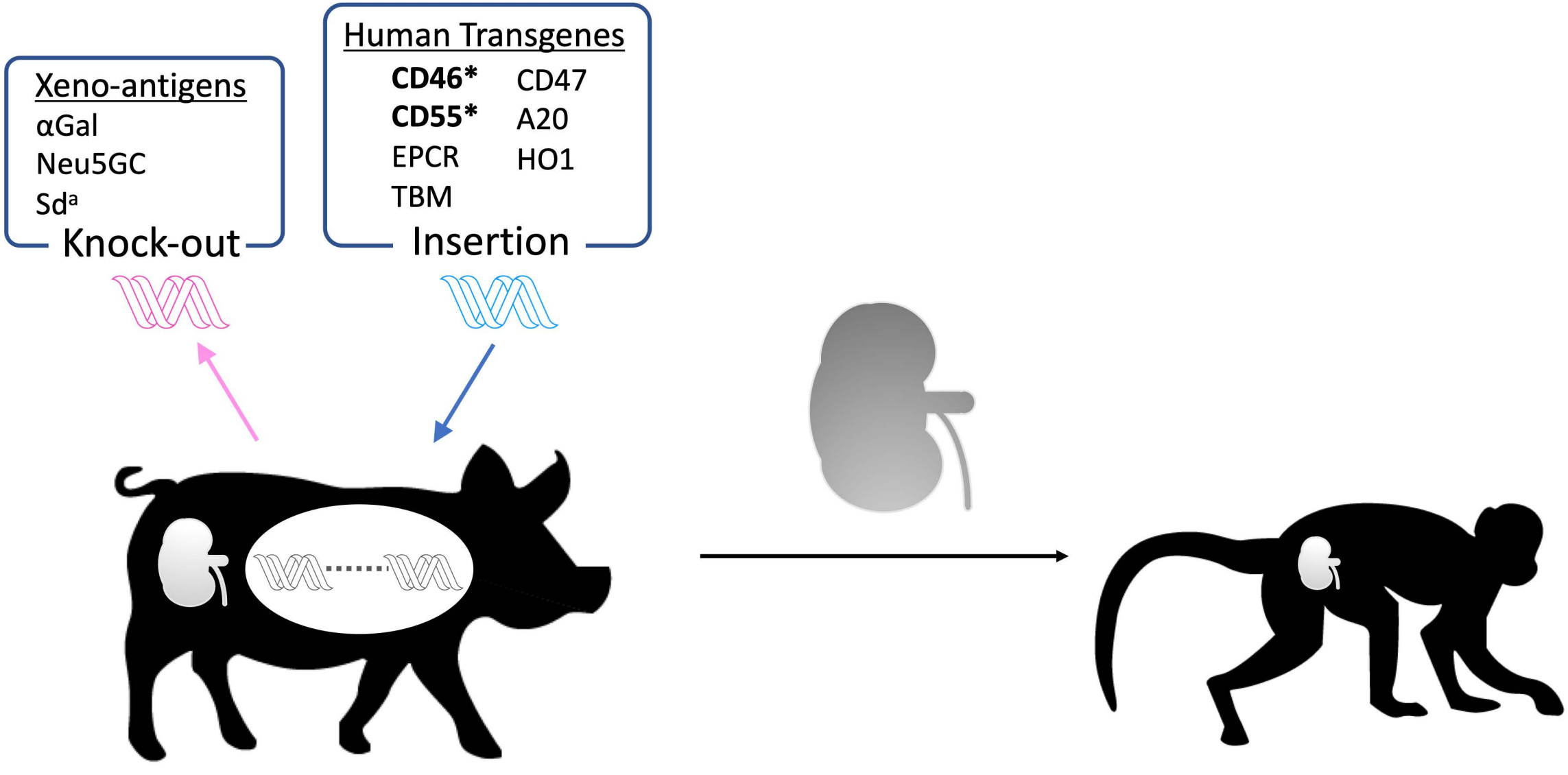
Figure 2. Recent preclinical advancements associated with gene modifications of donor pigs and complement inhibitors by chronological order. C1-INH, C1 esterase inhibitor; CVF, cobra venom factor; DKO, double-xeno antigen knock-out; GTKO, 1,3-galactosyltransferase knock out; IS, immunosuppressant; TG, transgenes; TKO, triple-xeno antigen knock-out;

## Figure 3 Caption

Figure 3. The kidney xenotransplantation model from gene-edited pigs to NHPs at MGH. Gene modification of donor pigs included complement regulatory proteins such as CD46 and CD55. *: Complement regulatory proteins; EPCR, endothelial protein C receptor; TBM, thrombomodulin.


DSA
IRI/Infection
IRI
Infection
Porcine endothelial cells
SLA
Neo-Ags
Xeno Ags
DAMPs
Abs
Bacterial surfaces
Mannose
PAMPs
Neo-Ags
MBL
MASPs
C1-INH
C1 complex (C1q/r/s)
Classical pathway
Lectin pathway
C2
C4
IRI
C3
C3b
Alternative pathway
C3bBb
C3 convertase
C3
C4bC2a
C3 convertase
Porcine-derived
Human/NHP-derived
Inhibition
C3a
C3b
C5 inhibitor
C5
C4bC2aC3b
C5 convertase
C5a
C5b
C6
C7
C8
C9
Terminal pathway
CRs
C5aR
C3aR
Immune cells
C5aR/C3aR
MAC
CD59
CD55
CD46
C4d
Porcine endothelial cells
Activation
Activation
Cell lysis
TCMR
IRI
AMR/TMA


GTKO
90 days
GTKO
CVF
Yamada et al.
(2005)
15 days
GTKO/CD55/
CD59/2TGs
C1-INH
Le Bas-Bernardet et al.
(2015)
260 days
GTKO/
CD55/CD46/
4TGs
Iwase et al.
(2017)
499 days
GTKO/CD55
Kim et al.
(2019)
35 days
CD55
Zaidi et al.
(1998)
51 days
CD55
Cozzi et al.
(2003)
90 days
CD55
Cavicchioli et al.
(2007)
DKO
557 days
DKO/
±SLA-1 KO
C5 inhibitor
Adams et al.
(2021)
68 days
CD55
C1-INH
Vangerow et al.
(2001)
TKO
61 days
TKO/
CD55/CD46/
4TGs
Yamamoto et al.
(2020)
69 days
CD46
Loveland et al.
(2003)
154 days
TKO/CD55/CD46/
4TGs
CVF
Ariyoshi et al.
(2021)
337 days
TKO/CD55/CD46/
5TGs
C1-INH
Eisenson et al.
(2023)
6 days
CD55/CD59
without IS
Ménoret et al.
(2004)
316 days
TKO/CD55/
CD46/CD59/
3TGs
Ma et al.
(2021)
758 days
TKO/
CD55/CD46/
5TGs
Anand et al.
(2023)
2000
2005
2010
2015
2020
2025
The longest survival days
Pig's gene modification
Complement inhibitory agents
Author
(Published year)


Xeno-antigens
αGal
Neu5GC
Sd^a
Knock-out
Human Transgenes
CD46* CD47
CD55* A20
EPCR HO1
TBM
Insertion