

Review

From Bench to Bedside: Immune and Genetic Innovations Driving the Future of Cardiac, Renal, and Hepatic Xenotransplantation

Running Title: Recent Advances of Xenotransplantation

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Abstract: Xenotransplantation, leveraging genetically engineered porcine donors, represents a promising solution to the global organ shortage crisis. Recent breakthroughs in genome editing have enabled the creation of pigs with multiple modifications, including knockout of key xenoantigens (*GGTA1*, *CMAH*, *B4GALNT2*) and insertion of human transgenes that regulate complement, coagulation, and innate immunity (e.g., *hCD46*, *hCD55*, *hTBM*, *hCD47*). Building on this genetic foundation, landmark clinical achievements have recently emerged. However, while the first porcine liver xenotransplants into human recipients demonstrated initial function, significant hurdles such as profound thrombocytopenia and coagulopathy—driven by factors like porcine vWF-human GPIb interactions and immune cell-mediated clearance—persist. Similarly, cardiac and renal xenotransplantation have seen milestones like the first pig-to-human heart transplants and extended kidney graft survival (up to 130 days) in a living recipient, yet delayed rejection and thrombotic microangiopathy remain critical challenges. Advanced strategies, including potent immunosuppression centered on anti-CD40 blockade, improved coagulation management (e.g., via *hTFPI*, *hEPCR*, *hCD39*), and emerging tolerance protocols, are actively being developed to overcome these barriers. This review synthesizes these pivotal 'bench-to-bedside' advancements, critically evaluating the current immunological and genetic innovations driving the progress of cardiac, renal, and hepatic xenotransplantation, and outlining the future directions necessary for successful clinical translation.

Keywords: xenotransplantation; genetic engineering; porcine donor; immune rejection; immunosuppression; coagulation disorders; complement regulation; clinical translation

1. Introduction

The global organ shortage crisis [1–4] necessitates alternative solutions, and xenotransplantation, the transplantation of organs between different species, has emerged as a promising novel strategy to potentially bridge this gap [5,6]. Among potential donor animals, pigs (porcine species) are considered particularly suitable candidates. This preference stems from several advantageous characteristics, including anatomical and physiological similarities to humans, relatively rapid reproduction rates allowing for scalable supply,

and notable biochemical parallels; for instance, porcine insulin is structurally almost identical to human insulin, differing by only two amino acids [7–11]. Therefore, based on these factors, porcine organs are often regarded as the most practical and suitable choice for xenotransplantation development.

Significant progress in xenotransplantation research, particularly since the 1990s, has been fundamentally enabled by advancements in genetic modification technologies applied to pigs. These modifications have been crucial in laying the foundation for potential clinical translation [12–17]. However,

the translation of xenotransplantation into clinical practice has historically been hindered by formidable biological barriers. Key challenges include immediate hyperacute rejection (HAR) [18–20], subsequent delayed xenograft rejection (DXR) involving both cellular and humoral mechanisms [21–28], the development of thrombotic microangiopathy (TMA) due to coagulation incompatibilities [29,30], and the potential, though debated, risk of transmitting porcine endogenous retroviruses (PERVs) to human recipients [7,31].

Current clinical progress in xenotransplantation is built upon decades of foundational research, primarily in large animal models. Seminal studies conducted in the 1990s, which focused on understanding immune tolerance, utilized nonhuman primate (NHP) models, and meticulously investigated xenoantigen rejection pathways, provided critical insights into the complex mechanisms underlying both acute and delayed rejection responses. These foundational studies paved the way for significant advancements in genetic engineering specifically aimed at overcoming these immunological hurdles. Specifically, the development of GalT knockout (*GalT*-KO) pigs, which lack the α -1,3-galactosyltransferase enzyme responsible for synthesizing the potent α -Gal xenoantigen, represented a major breakthrough in reducing the risk of HAR mediated by pre-existing anti-Gal antibodies in humans [32]. Subsequently, research efforts expanded to target other non- α -Gal carbohydrate antigens, such as N-glycolylneuraminic acid (Neu5Gc) and Sd(a), leading to the development of pigs with knockouts in the *CMAH* and *B4GALNT2* genes, respectively, further reducing the xenogeneic antigenic load [32].

Recent preclinical studies, often conducted in brain-dead human models, have begun to demonstrate the initial feasibility of transplanting these genetically modified porcine organs. For example, a 10-gene-edited porcine thymokidney, when transplanted into brain-dead recipients, maintained physiological function for 54 hours, showing improvements in estimated glomerular filtration rate (eGFR) ($23 \rightarrow 62$ and $55 \rightarrow 109$ ml/min/1.73 m²) and decreases in serum creatinine

($1.97 \rightarrow 0.82$ and $1.10 \rightarrow 0.57$ mg/dL). The graft produced substantial urine output (up to 744 ml/h), although proteinuria and hematuria were noted in one case, but importantly, there were no signs of antibody-mediated rejection [33]. Similarly, another study involving a renal xenograft in a brain-dead human demonstrated function for 77.5 hours. While day 1 biopsy showed some signs of TMA (glomerular congestion, endothelial swelling, fibrin thrombi), these were observed without deposition of C4d, IgG, IgM, or complement, and the glomerular structure appeared preserved by day 3 [29].

Moving towards living recipients, a landmark clinical case involved the first pig-to-human heart transplant performed in January 2022. This procedure utilized a 10-gene-edited pig heart from Revivicor, which included growth hormone receptor (*GHR*) knockout to control organ size, implanted into a 57-year-old patient with end-stage heart failure. The patient survived for 60 days with initially good graft function, although graft failure was ultimately attributed potentially to porcine cytomegalovirus (pCMV) reactivation [34]. Following this initial experience, a second cardiac xenotransplantation was performed by the same group, and further porcine kidney xenotransplantations have also occurred, experiences which will be elaborated upon later in this review. Significantly, in the realm of liver transplantation, a major advance was recently achieved by a Chinese group that successfully performed the first porcine liver xenotransplantation into a living human patient [35].

These pioneering successes in heart, liver, and kidney xenotransplantation, despite the remaining challenges, represent critical milestones and tangible breakthroughs for the field. Therefore, this review aims to systematically evaluate the current clinical and translational landscape of cardiac, renal, and hepatic xenotransplantation (Figure 1). By synthesizing recent advancements and ongoing challenges, we seek to provide insights into the trajectory of xenotransplantation as it progresses towards becoming a potential near-future therapeutic modality.

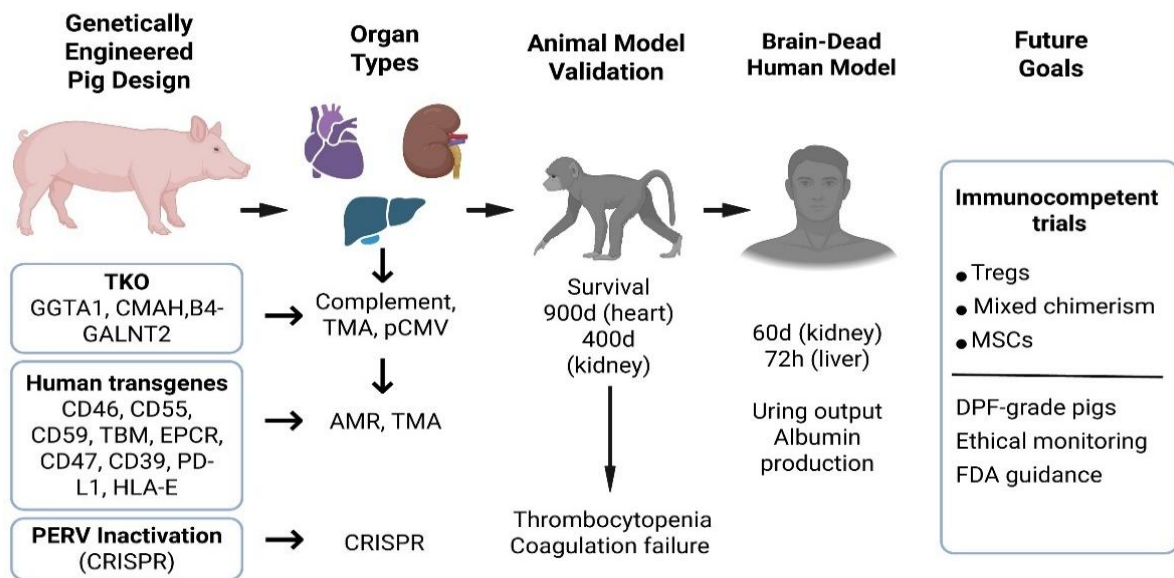


Figure 1. Roadmap of next-generation xenotransplantation.

This figure illustrates the translational pathway of xenotransplantation. Genetically modified Porcines feature triple xenoantigen knockout (*GGTA1*, *CMAH*, *B4GALNT2*), human transgenes (e.g., *CD46*, *CD55*, *TBM*, *EPCR*, *PD-L1*), and porcine endogenous retrovirus (PERV) inactivation via CRISPR to reduce immune rejection. Target organs include heart, kidney, and liver, addressing complement activation, antibody-mediated rejection (AMR), and thrombotic microangiopathy (TMA). Preclinical primate models show extended graft survival, while brain-dead human models demonstrate short-term function. Future goals include immunocompetent trials using regulatory T cells (Tregs), mixed chimerism, mesenchymal stem cells (MSCs), designated pathogen-free (DPF) Porcines, ethical oversight, and FDA regulatory alignment.

2. Liver Xenotransplantation

2.1. Main Barriers to Liver Xenotransplantation

Liver xenotransplantation faces distinct immunological and physiological challenges compared to cardiac and renal xenotransplantation [36]. This distinction arises partly from the liver's unique immunological environment; it is densely populated with resident antigen-presenting cells, such as Kupffer cells and liver sinusoidal endothelial cells (LSECs) [37], which readily interact with innate immune cells including natural killer (NK) and natural killer T (NKT) cells [38]. Furthermore, the liver itself is a major source of complement components, including C3 and C4 [39], potentially contributing significantly to local immune activation and injury post-transplantation.

Beyond the immune system, critical physiological incompatibilities also pose major barriers, particularly concerning hemostasis. Notably, porcine liver von Willebrand factor (vWF) exhibits a significantly higher affinity for the human platelet receptor GPIb compared to human vWF [13,40]. This species-specific interaction can lead to spontaneous and excessive platelet activation upon exposure to human blood. Coupled with inherent incompatibilities in other coagulation regulatory proteins between pigs and humans, which can trigger the extrinsic coagulation pathway, this high-affinity

vWF-GPIb binding often results in severe coagulopathy and profound thrombocytopenia. Moreover, these processes can fuel inflammation and further immune activation, involving CD8⁺ T cells, NK cells, and macrophages, post-xenotransplantation [13,40].

Immunologically, porcine livers, like other porcine tissues, express potent carbohydrate xenoantigens on their cell surfaces, including α -Gal, Neu5Gc, and Sd(a). These non-human glycans are readily recognized by preformed antibodies naturally present in most humans, significantly increasing the risk of both HAR and subsequent DXR [41,42]. To mitigate these immune responses, genetically modified pigs have been developed: *GGTA1*-knockout pigs lack α -Gal epitopes [43], *CMAH*-knockout pigs do not express Neu5Gc [43,44], and *B4GALNT2*-knockout pigs are deficient in Sd^a antigens [45]. These modifications aim to reduce the immediate threat of HAR, primarily triggered by anti- α -Gal antibodies, and also lessen the burden for delayed rejection, which can be exacerbated by antibodies targeting Neu5Gc and Sd(a). Consequently, combinations of these gene knockouts, particularly triple-knockout (TKO) pigs (*GGTA1*^{-/-}, *CMAH*^{-/-}, *B4GALNT2*^{-/-}), have been created to maximally minimize the binding of preformed human antibodies and reduce overall xenogeneic immune responses [46].

2.2. Early Clinical Experiences in Human Recipients

Recent years have witnessed the first attempts at clinical liver xenotransplantation using genetically modified porcine donors. In early 2024, a notable case involved Chinese surgeons performing porcine liver xenotransplantation using a four-gene-edited pig (with knockouts of *GGTA1*, *CMAH*, *β 4GalNT2*, and *GHR*) in a brain-dead 50-year-old male recipient. The graft demonstrated function for over 10 days. However, challenges persisted, attributed to responses against remaining non-Gal antigens, significant sequestration of platelets and leukocytes potentially mediated by Kupffer cells and LSECs, elevated inflammatory cytokines (e.g., IL-6, TNF- α), and activation of coagulation pathways possibly due to tissue factor exposure, thrombomodulin incompatibility, and macrophage/NK cell activation, all contributing to thrombocytopenia and inflammatory injury [47].

Another significant case was conducted by the First Affiliated Hospital of Anhui Medical University in a 71-year-old male patient with a hepatic tumor. This transplant utilized a more extensively modified 10-gene-edited pig liver (including *GGTA1*, *CMAH*, and *β 4GalNT2* knockouts, plus seven human transgenes aimed at immunomodulation and coagulation control). The graft reportedly showed immediate bile production upon reperfusion, along with increased synthesis of albumin and clotting factors. Encouragingly, there was no evidence of early rejection, and tests were negative for porcine cytomegalovirus, suggesting potentially lower innate immune activation and more stable liver sinusoidal endothelial cell function compared to earlier attempts [48].

A further advancement occurred in March 2025, when a team at the Fourth Military Medical University transplanted a six-gene-modified Bama miniature porcine liver into a living human recipient [35]. The xenograft began producing bile within 2 hours post-reperfusion, reaching a volume of 66.5 mL/day by day 10. Porcine albumin levels also rose significantly, reaching $\sim 3,000$ μ g/mL, indicating synthetic function. While liver enzymes showed a transient spike (AST peaked above 5,000 IU/L on day 1 before declining rapidly, ALT remained normal), early thrombocytopenia (platelet count $\sim 50\text{--}80 \times 10^9/\text{L}$) and prolonged APTT were observed, though these reportedly resolved. Importantly, histological examination revealed no signs of rejection. The immunosuppression regimen included tacrolimus, mycophenolate mofetil, methylprednisolone, with rituximab added later upon signs of B cell activation.

Building on these auxiliary and temporary approaches, surgeons at Xijing Hospital in China achieved another major

milestone in January 2025 by performing the world's first orthotopic (whole replacement) pig liver xenotransplantation into a brain-dead human recipient, completely replacing the native liver. Early reports indicated that the gene-edited pig liver successfully provided essential liver functions and showed signs of physiological stabilization (Chinese Academy of Sciences, ECNS.cn, 2025). This represents the first instance of complete native liver replacement with a xenograft in a human model, marking a conceptual advance over previous auxiliary support approaches.

2.3. Mechanistic Insights of Thrombocytopenia and Endothelial Activation and Functional Evaluation for Liver Xenotransplantation

Despite progress, liver xenotransplantation continues to face significant challenges, most notably the consistent occurrence of severe thrombocytopenia and coagulopathy, which can rapidly lead to early graft failure [49]. A primary molecular driver of these hemostatic complications is the significantly heightened affinity of porcine vWF for the human platelet glycoprotein Ib (GpIb) receptor [50,51], compared to the physiological interaction between human vWF and human GpIb. This high-affinity binding triggers excessive platelet activation and aggregation spontaneously upon contact with porcine endothelium or vWF, leading to their rapid consumption and the resulting thrombocytopenia [52,53]. Crucially, unlike its human counterpart which typically requires shear stress or endothelial injury for activation, porcine vWF can induce aggregation of human platelets directly, highlighting a key species incompatibility that predisposes to thrombosis [54,55].

In addition to vWF-mediated activation contributing to platelet loss, severe thrombocytopenia in liver xenotransplantation is further exacerbated by the active sequestration and phagocytosis of human platelets within the porcine liver sinusoids. This process is mediated by resident porcine liver cells, particularly LSECs and Kupffer cells, through pathways involving the asialoglycoprotein receptor 1 (ASGR1), CD40 signaling, and potentially blockade of the "don't eat me" signal via the SIRP α /CD47 interaction [40,56]. The hepatic sinusoidal endothelium appears central to initiating this widespread platelet activation and consumption, primarily via the high-affinity vWF–GPIb binding, and also contributes significantly to platelet clearance through phagocytosis involving ASGR1 and possibly CD40–CD154 interactions [57,58]. Furthermore, porcine Kupffer cells and LSECs actively contribute not only to platelet phagocytosis but also exacerbate coagulopathy by releasing procoagulant factors,

including additional VWF and Factor VIII, into the local environment [59,60].

The persistence of these profound consumptive coagulopathies, even in the context of genetically modified donors, remains a major obstacle in liver xenotransplantation, underscoring the intricate and multifactorial nature of the underlying mechanisms [61,62]. Indeed, even with the introduction of human transgenes aimed at improving anticoagulation, such as human thrombomodulin (*hTM*) and human endothelial protein C receptor (*hEPCR*), these complications have often remained inadequately controlled in preclinical models and early human studies [63]. This observation highlights the potential need for incorporating additional or different anticoagulant or immunomodulatory transgenes, such as human tissue factor pathway inhibitor (*hTFPI*) to dampen extrinsic coagulation, or human CD47 (*hCD47*) to inhibit phagocytosis [64].

Adding to the complexity of cell-cell interactions driving pathology, signaling between porcine liver immune cells and activated human cells can worsen thrombocytopenia and inflammation. Specifically, CD40–CD40L signaling, occurring between porcine Kupffer cells (expressing CD40) and activated human platelets (which upregulate CD40L), appears to further promote platelet phagocytosis and clearance by Kupffer cells [36,49,65]. This deleterious pathway can be significantly amplified in the inflammatory milieu typically associated with transplantation, as inflammation often leads to increased CD40L upregulation on platelets and other immune cells [52,66], creating a positive feedback loop.

To address these multifaceted challenges, various strategies are being explored. Genetic modifications offer potential solutions at the source. For instance, expressing a 'humanized' form of vWF in pigs, engineered to have lower affinity for human GPIb, has shown promise in reducing platelet activation during *ex vivo* perfusion studies [50]. As mentioned, expressing human TM and EPCR aims to improve anticoagulation by enhancing the protein C pathway [63]. Moreover, incorporating *hTFPI* aims to suppress the initiation of coagulation via the tissue factor/Factor VIIa (TF/FVIIa) complex and also inhibit Factor Xa (FXa) through its Kunitz domains, thereby prolonging clotting times [67]. Expressing *hCD47* on porcine cells aims to provide a crucial “don't eat me” signal to human phagocytes (like macrophages and Kupffer cells) by engaging their SIRPα receptor, thus limiting immune cell-mediated clearance of graft cells and potentially platelets [68].

Beyond genetic engineering of the donor, procedural approaches are also being investigated. Preconditioning porcine livers using normothermic machine perfusion with

human plasma before transplantation has been shown to reduce endothelial activation markers, lower levels of vWF, IL-6, and TNF-α release, and potentially improve immediate graft compatibility upon reperfusion [69,70]. Another innovative strategy, though technically challenging, involves attempting to engineer chimeric endothelia using porcine blood-derived endothelial-like cells modified to express key human endothelial markers like CD31, VE-Cadherin, alongside controlled vWF expression, with the goal of reducing the thrombogenicity of the graft vasculature [71].

Functionally, despite the challenges, porcine livers transplanted into various preclinical models have demonstrated the capacity for essential hepatic tasks. These include producing albumin and key clotting factors (like II, VII, X), metabolizing bilirubin [41], and secreting bile [72]. In both *ex vivo* perfusion systems and *in situ* transplantations into NHPs or brain-dead human recipients, porcine livers have consistently shown evidence of initial functionality, including bile production [72], albumin production [69], and the ability to maintain portal venous pressure and hepatic artery flow [73,74]. However, this initial period of function is often distressingly short-lived, with rapid decline typically observed within hours to days (often less than 72 hours). This early failure is predominantly attributed to the rapid onset of consumptive coagulopathy, characterized histologically by widespread platelet aggregation, fibrin deposition within sinusoids, and evidence of macrophage-mediated platelet phagocytosis involving pathways like CD18, vWF-GPIb interactions, and ASGR1. Immune-mediated endothelial injury, triggered by preformed non-Gal antibodies binding to the endothelium, and potential issues arising from cross-species incompatibility in innate immune signaling (such as the CD47–SIRPα pathway) also likely contribute to this early functional deterioration [75].

3. Cardiac Xenotransplantation

3.1. Preclinical and Clinical Milestones

Cardiac xenotransplantation research has progressed significantly, driven largely by extended graft survival achieved in preclinical NHP models. A pivotal study demonstrating this progress was conducted by Mohiuddin et al., involving heterotopic (auxiliary) transplantation of hearts from genetically modified pigs into baboons. The donor pigs possessed an α1,3-galactosyltransferase gene-knockout (*GTKO*) background and were engineered to express two human proteins: the complement-regulatory protein CD46 (*hCD46*) and the anticoagulant protein thrombomodulin (*hTBM*). The

recipient baboons received a specifically designed immunosuppressive regimen centered on costimulation blockade, comprising an anti-CD40 monoclonal antibody (mAb) (20 mg/kg initially, then 10 mg/kg weekly), alongside mycophenolate mofetil (MMF) (500 mg twice daily) and corticosteroids (tapered dose). This combination of genetic modification and targeted immunosuppression resulted in remarkable graft survival, extending up to 945 days, with a mean survival of 433 days, marking a substantial advancement for the field [76].

Building on heterotopic success, efforts moved towards orthotopic (life-supporting) cardiac xenotransplantation. Långin et al. achieved noteworthy outcomes by transplanting hearts from *GTKO* pigs expressing *hCD46* and *hTBM* orthotopically into baboons. Their immunosuppressive regimen also utilized anti-CD40 mAb (though at a different dosing: 50 mg/kg initially, then 20 mg/kg weekly), combined with MMF (500 mg twice daily) and corticosteroids (similar taper). This approach led to life-supporting graft function and recipient survival for up to 195 days, with a mean survival of 90 days [77]. Confirming the potential for long-term survival, Mohiuddin et al. also later reported achieving over 945 days of heterotopic cardiac xenograft survival in baboons using refined immunosuppression regimens involving anti-CD40 mAbs and costimulation blockade strategies [78].

The success of these prolonged xenograft survivals is attributed directly to the synergistic effect of specific genetic modifications in the donor pig and the tailored immunosuppressive protocols used in the recipient. The *GTKO* modification is fundamental, as it eliminates the α -Gal epitope, thereby preventing HAR mediated by pre-formed anti-Gal antibodies [79,80]. Expression of *hCD46* and *hTBM* on the donor porcine endothelial cells provides crucial additional protection. *hCD46* enhances regulation of the human complement cascade by acting as a cofactor for Factor I-mediated inactivation of C3b and C4b, while *hTBM* promotes anticoagulation by binding thrombin and enhancing the activation of the protein C pathway. Together, these modifications help reduce platelet aggregation, limit thrombin generation, protect the endothelium from complement-mediated injury, and thereby mitigate the risk of TMA, a common failure mode in lung and cardiac xenografts often linked to dysregulation of the vWF–GPIb and thrombin–protein C axes [81,82]. Crucially, the immunosuppressive regimens, particularly the use of anti-CD40 monoclonal antibodies, have proven highly effective. By blocking the critical CD40–CD154 costimulatory pathway necessary for full T-cell activation and B-cell help, these agents prevent T-cell-

mediated rejection and antibody production, while importantly avoiding the thromboembolic complications that plagued earlier attempts using anti-CD154 antibodies [83,84].

These preclinical successes paved the way for clinical attempts. A landmark clinical case, as previously mentioned, involved a 57-year-old patient with end-stage heart failure who received a heart transplant from a genetically modified porcine donor in January 2022 [34]. The donor pig incorporated ten specific genetic modifications designed to maximize compatibility and minimize rejection. These included the knockout of three key carbohydrate antigen genes (*GGTA1*, *CMAH*, and *B4GALNT2*) to prevent antibody binding, and the insertion of six human transgenes intended to regulate immune acceptance and coagulation pathways (*hCD46*, *hCD55*, *hCD59*, *hEPCR*, *hTHBD* [encoding TBM], and *hCD47*). Additionally, the pig's growth hormone receptor (*GHR*) gene was inactivated to prevent excessive organ growth post-transplantation. The patient survived for 60 days with initially good graft function, demonstrating the feasibility of the approach [34].

Subsequently, a second genetically modified porcine heart transplant was performed in late 2023 on a 58-year-old patient with terminal heart failure, again at the University of Maryland Medical Center. This procedure also utilized a 10-gene-edited porcine donor heart. The recipient was selected based on criteria including better physiologic reserve and low levels of preformed anti-porcine antibodies. Immunosuppression in this case featured Tegoprubart, a novel non-depleting anti-CD40L (CD154) monoclonal antibody undergoing clinical trials, aiming to potentially inhibit T-cell activation and antibody-mediated rejection pathways. Despite initially favorable graft function, the xenograft eventually developed progressive endothelial injury. Pathological analysis revealed features indicative of antibody-mediated rejection, including capillary deposition of C3d, C4d, IgG, and IgM, along with infiltration by CD68⁺ macrophages. The complement blockade strategy was transitioned from C1 esterase inhibitor (Berinert) to C5 inhibition (Eculizumab) during the course. Notably, detailed transcriptomic analysis of the explanted failing graft revealed significant overexpression of immune-related genes, including *HLA-A*, *CD74*, *CIQA*, *CXCL9*, and *FCGR3A*, reflecting an intense underlying humoral and innate immune response against the xenograft. These findings underscore the complexity of controlling rejection in pig-to-human xenotransplantation, highlighting the critical need for enhanced immunomonitoring tools, multimodal graft assessment (phenotyping), and potentially further refinement of transgene combinations or immunosuppressive strategies to improve long-term outcomes [85]. Notably, anti-CD40 monoclonal

antibodies have entered early-phase clinical trials in transplantation and have shown a favorable safety profile, but published data in xenotransplantation remain limited.

3.2. Immunological and Hemostatic Barriers

Despite the significant progress enabled by genetic engineering and advanced immunosuppression, cardiac xenografts continue to face complex immunological and hemostatic challenges, which are schematically represented in Figure 2. Hyperacute rejection, once the primary immediate barrier, is now largely preventable through the knockout of the *GGTA1* gene. This modification effectively removes the α -Gal epitope, the main target for natural human IgM and IgG antibodies that trigger the classical complement cascade [86–88]. However, even with HAR controlled, the subsequent phase of delayed xenograft rejection (DXR) remains a significant problem. DXR is understood to be a multifaceted process mediated by a combination of factors including elicited antibody responses against non-Gal xenoantigens, subsequent antibody-dependent complement activation on the endothelium, endothelial cell activation leading to a pro-inflammatory and pro-coagulant state, and infiltration by innate immune cells, particularly monocytes/macrophages [22,89].

To combat complement-mediated injury contributing to DXR, the expression of human complement regulatory proteins like *hCD46* and *hCD55* on porcine endothelial cells is crucial. *hCD46* functions as a cofactor for Factor I-mediated inactivation of deposited C3b and C4b, thereby dampening complement amplification [90]. *hCD55* (decay-accelerating factor) accelerates the breakdown of the C3 and C5 convertase enzyme complexes, further limiting complement activation [90,91]. Expression of *hCD59* (protectin), often included in multi-transgene strategies, directly inhibits the formation of the terminal membrane attack complex (MAC), protecting cells from lysis.

Coagulation incompatibility represents another critical hurdle. Porcine endothelial cells, when exposed to human blood and inflammatory mediators, tend to become pro-coagulant. They may express tissue factor and activate coagulation cascades more readily than human endothelium, partly due to species incompatibilities in natural anticoagulant mechanisms, predisposing the graft to thrombotic microangiopathy (TMA) [92]. This risk is mitigated by expressing human anticoagulant proteins like *hTBM* and human endothelial protein C receptor (*hEPCR*) in the donor pig. *hTBM* binds thrombin, converting it into an activator of the protein C pathway, while *hEPCR* significantly enhances this activation [93,93]. Activated protein C then degrades pro-coagulant

factors Va and VIIIa, providing a negative feedback loop on coagulation. Despite these efforts, residual platelet activation and microthrombi formation can persist. Therefore, the inclusion of *hCD39*, an ectonucleotidase that hydrolyzes pro-thrombotic ATP and ADP released from activated platelets or damaged cells into AMP, is increasingly being used as an additional strategy to inhibit platelet aggregation and help maintain graft microvascular patency [95].

The implementation of immunosuppressive regimens based on anti-CD40 monoclonal antibodies has been pivotal in achieving the long-term preclinical survivals observed [96]. By specifically targeting the CD40–CD154 (also known as CD40L) co-stimulatory pathway, which is essential for T cell-dependent B cell activation and optimal T cell priming, these agents effectively inhibit key adaptive immune responses. This blockade reduces detrimental cellular infiltration and antibody production, thereby preserving graft integrity [83,97]. Finally, another important transgene addition is *hCD47*. This molecule interacts with the signal regulatory protein alpha (SIRP α) receptor expressed on human macrophages and other myeloid cells. This interaction delivers an inhibitory “don’t eat me” signal, reducing the innate immune system’s ability to clear graft endothelial cells via phagocytosis [98,99]. As illustrated in Figure 2, these combined genetic strategies (targeting antigens, complement, coagulation, innate immunity) and molecular immunosuppression (targeting adaptive immunity) have synergistically extended graft survival in preclinical NHP studies and form the basis of protocols currently being translated into early-phase human xenotransplantation trials.

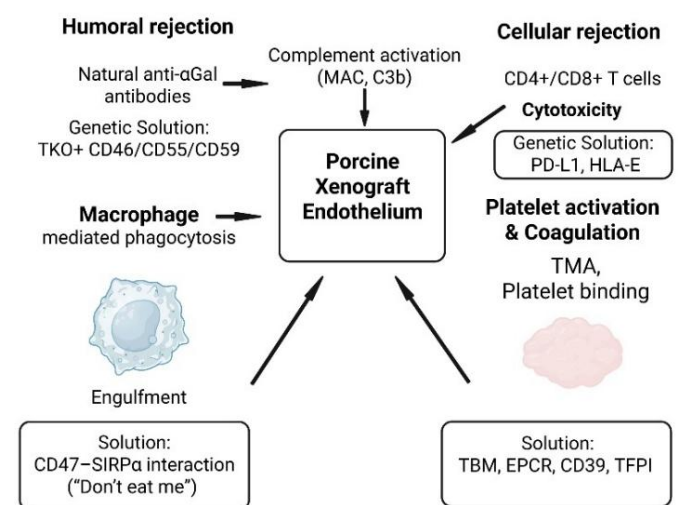


Figure 2. Key immunologic and hemostatic barriers in porcine xenograft endothelium and corresponding genetic solutions. This schematic highlights major immune and coagulation-related challenges faced by porcine endothelial grafts. Humoral rejection involves natural anti- α Gal antibodies and complement

activation (membrane attack complex [MAC], C3b), addressed through triple knockout (TKO) and expression of human complement regulators CD46, CD55, and CD59. Cellular rejection is mediated by cytotoxic CD4⁺/CD8⁺ T cells, mitigated by the expression of immune checkpoint molecules PD-L1 and HLA-E. Macrophage-driven phagocytosis is counteracted by introducing CD47 to engage SIRP α and deliver a “don’t eat me” signal. Platelet activation and thrombotic microangiopathy (TMA) are reduced by expressing thrombomodulin (TBM), endothelial protein C receptor (EPCR), CD39, and tissue factor pathway inhibitor (TFPI).

3.3. Functional Assessments in Preclinical Models

Functional evaluation in NHP models has been critical for demonstrating the potential of cardiac xenotransplantation. Orthotopic transplantation of genetically modified porcine hearts into baboons has successfully shown the ability of these xenografts to sustain life-supporting cardiac output over extended periods [100–102]. Notably, as mentioned previously, porcine hearts with combined *GTKO/hCD46/hTM* modifications, when transplanted into baboons receiving anti-CD40-based immunosuppression supplemented with mTOR inhibition (using temsirolimus), achieved survival up to 195 days. Importantly, these hearts maintained preserved cardiac function with minimal signs of immune infiltration throughout the study period [77].

Advanced imaging modalities, such as echocardiography and cardiac magnetic resonance imaging (MRI), have been instrumental in non-invasively evaluating xenograft function and structure in these preclinical models [103]. Serial echocardiographic assessments in successful long-term NHP studies typically revealed preserved left ventricular ejection fraction (LVEF) and normal chamber dimensions. These findings were consistent with early graft stability, which was often supported by laboratory findings of low anti-pig IgG/IgM antibody titers, minimal C4d deposition (a marker of classical complement activation) on biopsy, and histological evidence of intact endothelial morphology [104,105]. MRI studies further corroborated the preserved graft status in some long-term survivors, showing minimal evidence of adverse vascular remodeling or intimal thickening, and correlating with limited infiltration of pro-inflammatory IFN- γ ⁺ T cells and low expression of vascular endothelial growth factor A (VEGF-A), suggesting relative immune quiescence [106].

Histopathological analysis of long-term surviving cardiac xenografts (e.g., >1 year in heterotopic models) often reveals minimal evidence of active inflammatory cell infiltration. Common findings include low levels of portal and lobular

inflammation (if applicable, though less relevant for heart), a general absence of classical T cell-mediated rejection features like lymphocytic infiltrates attacking myocytes, and lack of significant endothelialitis or biliary injury (relevant for liver, not heart) [107]. When inflammatory infiltrates are present, especially during episodes of rejection or dysfunction, they often predominantly consist of macrophages and natural killer (NK) cells, with sometimes surprisingly sparse T-cell involvement, particularly under effective costimulation blockade [108].

Macrophages are thought to contribute significantly to xenograft rejection through multiple mechanisms. They can produce cytotoxic mediators such as TNF- α , IL-1, and nitric oxide, directly damaging graft tissue. They also act as antigen-presenting cells, potentially activating xenoreactive CD4⁺ T cells by presenting porcine peptides and secreting cytokines like IL-12 [109,110]. Furthermore, activated macrophages secrete a range of pro-inflammatory cytokines including TNF- α , interleukin-1 (IL-1), and interleukin-6 (IL-6), which can amplify the inflammatory response by inducing endothelial activation and promoting further recruitment of leukocytes into the graft [111,112].

NK cells represent another key innate immune barrier. They can mediate direct cytotoxicity against porcine endothelial cells through the release of perforin and granzymes, leading to apoptosis [113,114]. NK cell activation in the xenogeneic setting can be triggered by mismatches in MHC molecules. For instance, the interaction (or lack thereof) between porcine MHC class I (termed SLA-I) molecules and human NK cell inhibitory receptors (like Killer Immunoglobulin-like Receptors [KIRs] or receptors like NKG2A/CD94 and ILT2) can lead to NK cell activation due to “missing-self” recognition or recognition of specific porcine ligands. NK cell degranulation, a marker of cytotoxic activity, can be assessed by measuring CD107a upregulation [115,116]. Conversely, strategies to overcome NK cell rejection include genetic modifications to express human MHC molecules like HLA-E or HLA-G on pig endothelial cells. These human MHC molecules can engage inhibitory receptors (like NKG2A or ILT2) on human NK cells, thereby suppressing NK cytotoxicity [115,116].

4. Renal Xenotransplantation

4.1. Clinical Milestones in Brain-Dead Human Models

Between 2021 and 2023, several institutions, notably NYU Langone Health and the University of Alabama at Birmingham (UAB), conducted pivotal studies involving the transplantation

of porcine kidneys from genetically modified donors into brain-dead human recipients [29,33]. These experiments provided crucial proof-of-concept data in a human physiological context without risks to living patients. The donor pigs used in these studies were typically engineered with triple knockout (TKO) modifications—disrupting the *GGTA1*, *CMAH*, and *B4GALNT2* genes—in order to eliminate the major carbohydrate xenoantigens responsible for triggering hyperacute rejection. Additionally, various human transgenes, such as *hCD46* and *hCD55*, were often included to provide better regulation of the human complement cascade upon reperfusion and enhance initial graft survival [117].

A landmark study by the NYU Langone Health group, reported in 2023, involved transplanting a single gene-edited (*GGTA1*-KO only) porcine kidney into a brain-dead recipient. Remarkably, this kidney maintained physiological function, including robust urine production and creatinine clearance, for a total of 61 days post-implantation. This marked the longest duration of functional porcine kidney xenograft survival reported at that time [118,119]. The graft produced urine almost immediately after reperfusion, and serum creatinine levels normalized within three days, remaining stable for over 50 days. Histological analyses confirmed the absence of hyperacute rejection. However, evidence of mild thrombotic microangiopathy (TMA) and endothelial activation—characterized by increased endothelial expression of von Willebrand factor and adhesion molecules—was observed. Interestingly, despite the donor pig lacking human complement regulatory transgenes like *hCD55* or *hCD46*, the graft was not acutely rejected, suggesting that perhaps some degree of immune accommodation occurred, potentially facilitated by the intensive triple immunosuppression regimen used, which might have suppressed NK cell cytotoxicity (evidenced by reduced CD107a⁺ NK cell infiltration) [33].

Building upon these brain-dead model studies, the first transplant of a gene-edited pig kidney into a living patient occurred in late 2024. Surgeons successfully transplanted a kidney from a pig with 10 specific genetic modifications—including deletion of the three main carbohydrate antigens (*GGTA1*, *CMAH*, *B4GALNT2*) and insertion of seven human transgenes (likely including complement regulators, coagulation modulators like *hTBM/hEPCR*, and potentially *hCD47* and others)—into a living patient deemed to have high antibody sensitivity, making conventional allotransplantation difficult. The graft reportedly functioned immediately, producing urine and clearing creatinine, and supported stable renal function for an impressive 130 days. This achievement marked the longest survival of a porcine kidney xenograft in a

living human to date and demonstrated substantial immunologic compatibility under a standard immunosuppression regimen (AP News, 2024). However, in April 2025, the recipient, 53-year-old Towana Looney, ultimately required explantation of the pig kidney after 130 days due to the development of irreversible graft dysfunction, likely from a form of delayed rejection. Although she returned to dialysis, her case represented a significant milestone in demonstrating extended function and provided invaluable insights into the challenges of late-phase xenograft immune rejection and the limitations of current immunosuppression protocols in preventing it (Science, 2025).

These pioneering clinical efforts build upon foundational research dating back to the 1990s. Key early work included the introduction of α -Gal knockout pigs to overcome HAR mediated by natural anti-Gal antibodies, and experiments exploring the induction of donor-specific tolerance using strategies like mixed hematopoietic chimerism. Mixed chimerism aims to establish coexistence of donor and recipient hematopoietic cells, potentially leading to tolerance by deleting reactive T and B cells through central (thymic selection) and peripheral (clonal deletion or anergy) mechanisms [120]. More recently, sophisticated analyses like single-cell and spatial transcriptomic profiling have been applied to porcine kidney xenografts transplanted into brain-dead human models. These studies revealed relatively limited infiltration of human immune cells into the renal cortex during the early post-transplant period. The few human immune cells detected were predominantly CD14⁺ monocytes and CD68⁺ macrophages, many of which exhibited high expression of anti-inflammatory markers such as *IL10*, *MRC1*, and *CD163*, suggesting a potentially suppressed or polarized immune environment. Notably, human CD3E⁺ T cells and CD19⁺ B cells were largely undetectable in the graft tissue during these early phases under immunosuppression [121].

Similarly, the UAB group conducted a study in 2023 where kidneys from 10-gene-edited pigs were transplanted into a brain-dead human recipient. The immunosuppression regimen included induction with anti-thymocyte globulin (ATG), rituximab (anti-CD20 B cell depletion), and eculizumab (C5 complement blockade). The grafts produced urine within just 4 minutes of reperfusion and maintained function throughout the 7-day study period. Serial biopsies showed well-preserved tubular epithelial structures, a notable absence of CD61⁺ platelet thrombi (suggesting TMA control), and minimal immune cell infiltration. This favorable outcome was attributed to the protective effects conferred by the specific combination of transgenes expressed in the donor kidney, likely including

complement regulators (*hCD55*), anti-phagocytic signals (*hCD47*), and potentially anti-inflammatory genes like heme oxygenase-1 (*HMOX1*) [122].

4.2. Preclinical Validation in Non-Human Primates

Prior to human studies, extensive preclinical validation occurred in NHP models. Both orthotopic (life-supporting) and heterotopic (auxiliary) renal xenotransplantation using genetically modified porcine kidneys demonstrated encouraging outcomes in NHPs (typically baboons or cynomolgus macaques). Graft survival durations commonly ranged from 30 to 90 days, with several isolated reports achieving survival exceeding 180 days, and even reaching beyond 400 days in specific cases [123–126]. For instance, remarkable long-term survival of renal xenografts (beyond 400 days) was achieved in NHPs when recipients were preselected for low levels of pre-existing anti-pig IgG antibodies and received an immunosuppressive regimen including anti-CD4 mAb, the anti-CD154 mAb 5C8 (costimulation blockade), mycophenolate mofetil (MMF), and corticosteroids (solumedrol). This combination effectively suppressed CD4⁺ T cell-mediated rejection and significantly delayed the onset of antibody-driven graft injury [127].

Functionally, successful grafts in these NHP studies consistently demonstrated the ability to support life or maintain physiological parameters. This included normalization of serum creatinine levels (in orthotopic models), sustained urine production (diuresis), and maintenance of stable electrolyte homeostasis [128]. These successful long-term outcomes were often critically dependent on strategies aimed at inducing tolerance or providing potent immunosuppression. Examples include co-transplantation of vascularized donor thymus tissue to promote mixed chimerism and central tolerance, or the use of robust costimulation blockade agents targeting either the CD154 or CD40 pathway [128]. Histopathological analysis of long-surviving grafts frequently revealed well-preserved renal architecture with minimal tubular injury and notably low levels of cellular infiltrate, particularly when potent anti-CD40-based immunosuppression was employed [129].

A key factor enabling prolonged graft survival in many recent NHP studies has been the incorporation of costimulatory blockade agents, especially monoclonal antibodies targeting CD40. One such agent used successfully is 4D11, a fully human anti-CD40 IgG4 monoclonal antibody. Regimens based on this antibody effectively inhibited T-cell costimulation (by blocking CD40 signaling required for APC licensing) and B-cell activation and antibody production (as CD40 signaling is critical for T-dependent B cell responses) without triggering the

thromboembolic events that were unfortunately associated with earlier generations of anti-CD154 agents [130].

Standard immunosuppressive protocols used in these NHP studies often mirrored clinical practice but were typically more intensive. For example, methylprednisolone might be administered intravenously at high doses initially, followed by tapering to oral dosing, in order to suppress early inflammatory cytokine production and broad leukocyte activation [131]. MMF is usually administered orally; it functions by targeting inosine monophosphate dehydrogenase (IMPDH), an enzyme crucial for the *de novo* synthesis of guanine nucleotides. Since lymphocytes are heavily reliant on this pathway for proliferation, MMF effectively dampens both T- and B-cell responses [132]. Tacrolimus, another cornerstone agent (though sometimes replaced by costimulation blockade), is typically administered orally with dose adjustments based on trough blood levels. As a calcineurin inhibitor, it blocks T-cell activation primarily by preventing the dephosphorylation and nuclear translocation of the transcription factor NFAT, which is required for the transcription of IL-2 and other key T-cell activation genes [133].

4.3. Immunological and Coagulation Challenges

Despite the significant advances achieved through genetic modification of porcine donors to eliminate major xenoantigens (like α -Gal, Neu5Gc, Sd(a)), renal xenografts continue to face persistent challenges related to delayed forms of rejection. These include antibody-mediated rejection (AMR) and T cell-mediated rejection (TCMR), although the latter is often better controlled by modern immunosuppression [20,134–136]. Histopathological analyses from both NHP models and the human brain-dead recipient studies have frequently documented the deposition of C4d along peritubular capillaries. C4d deposition is considered a hallmark indicator of classical complement pathway activation, often initiated by antibody binding, and is thus suggestive of AMR. This finding is often accompanied by other histological signs of microvascular inflammation, such as focal glomerulitis characterized by endothelial swelling and infiltration of mononuclear cells into the glomeruli [137–139]. Furthermore, flow cytometry assessments of recipient serum have often detected the persistence or *de novo* development of donor-specific antibodies (DSAs) post-transplantation, even in recipients treated with B-cell depleting agents like rituximab. This underscores the resilience of humoral responses and the need for effective, targeted immunosuppressive strategies specifically aimed at controlling antibody production and B cell memory [140].

Intravascular coagulation also remains a significant concern in renal xenotransplantation, manifesting often as TMA [65]. Even with TKO modifications and the introduction of human complement and coagulation regulatory genes—such as *hEPCR* [63] and *hTBM* [141]—instances of microthrombi formation within glomerular capillaries and arterioles, characteristic of TMA, have been frequently observed histologically. This tends to become apparent particularly in the weeks following transplantation (often between 2 to 4 weeks post-transplant in NHP models) [142]. To further address both immune-mediated injury and coagulation issues at the glomerular level, ongoing research utilizes additional transgenes. For example, expressing *hCD47* specifically on porcine glomerular endothelial cells and podocytes aims to engage the recipient's SIRP α receptor on infiltrating macrophages [143], thereby inhibiting phagocytic clearance of these crucial cells and potentially reducing proteinuria. Co-expression of *hCD39* aims to degrade extracellular ATP released during injury, thereby limiting P2 receptor-mediated platelet activation and dampening microvascular thrombosis [144].

Addressing these persistent immunological and coagulation challenges is therefore critical for the future success and clinical advancement of renal xenotransplantation. Current strategies focus on several fronts. These include incorporating a broader array of human coagulation-regulatory genes into donor pigs, such as *hCD39*, *hTBM*, *hEPCR*, and *hTFPI*, to more comprehensively mitigate thrombosis risk. Simultaneously, more effective approaches for targeting B-cell activation and DSA production are needed. Importantly, however, some studies suggest the intrinsic coagulation issues might be less severe in kidney compared to liver. For example, experiments involving perfusion of porcine kidneys with human platelets labeled with CFSE, followed by confocal microscopy examining CD31⁺ endothelial cells, indicated that human platelets did not significantly bind to or become consumed by porcine renal endothelial cells in the absence of immune injury [65,145].

4.4. Functional Biomarkers and Graft Monitoring

Monitoring the function and health of a renal xenograft after transplantation necessitates a comprehensive approach. This should integrate traditional clinical markers with emerging, potentially more sensitive biomarkers in order to detect early signs of graft dysfunction or rejection, ideally before irreversible damage occurs. Standard parameters such as serum creatinine concentration, blood urea nitrogen (BUN) level, and urine output remain foundational tools for assessing overall

renal function [122,146]. However, a major limitation of these markers is that they often reflect established injury rather than predicting it; significant changes may only occur after substantial graft damage has already taken place, thus lacking the sensitivity needed to identify subclinical injury promptly. This limitation prompts the exploration and validation of novel biomarkers for xenograft monitoring.

One such potential biomarker is urinary neutrophil gelatinase-associated lipocalin (NGAL). NGAL is a 25-kDa protein that is rapidly synthesized and released by injured renal tubular epithelial cells [147]. Elevated urinary NGAL levels have been strongly associated with acute kidney injury (AKI) in various clinical settings [148], and it holds promise as an early indicator of graft dysfunction or injury in the transplant setting [149]. Studies in allotransplantation have demonstrated that urinary NGAL levels can rise significantly within hours of renal injury, often preceding significant increases in serum creatinine by 24–48 hours [150]. For instance, in human kidney transplant recipients, elevated urinary NGAL has been linked to outcomes like delayed graft function (DGF) and episodes of acute rejection [151,152], suggesting its potential utility in xenotransplantation monitoring, although species-specific assay validation would be required.

Donor-derived cell-free DNA (dd-cfDNA) represents another highly promising biomarker for non-invasive graft monitoring [153,154]. dd-cfDNA consists of small DNA fragments released into the recipient's bloodstream from dying graft cells. Elevated levels of dd-cfDNA in the recipient's plasma or urine can serve as a quantitative indicator of graft injury, regardless of the cause (e.g., rejection, ischemia, infection) [155]. Quantification of the fraction of total cfDNA that is donor-derived allows for sensitive and specific detection of allograft injury. In allotransplantation, increased dd-cfDNA levels have been shown to correlate well with biopsy-proven rejection, often rising before clinical signs or changes in traditional markers become apparent [156]. This approach enables the possibility of early intervention, potentially mitigating graft damage and improving long-term outcomes [157]. Its application to xenotransplantation is feasible, requiring assays that can distinguish porcine from human cfDNA.

Imaging modalities also play a crucial role in the routine assessment of transplanted kidneys. Doppler ultrasound, for example, is a non-invasive technique used to evaluate renal perfusion and vascular integrity. It can detect changes in blood flow patterns (e.g., increased resistive indices) that may be indicative of vascular complications, rejection, or other causes of graft dysfunction [158,159]. Alterations in resistive indices

measured by Doppler ultrasound have been associated with acute rejection and other forms of graft pathology in allotransplantation [160]. Therefore, regular monitoring using Doppler ultrasound can aid in the early detection of potential complications, guiding the need for further investigation (like biopsy) or timely therapeutic interventions [161].

While kidney xenotransplantation has shown considerable promise, particularly in recent preclinical NHP and brain-dead human model studies, significant immunological and hemostatic challenges undeniably remain. Early functional success in some models has been associated with effective modulation of immune responses, for example, showing reduced C3b/C5b-9 deposition and lower levels of inflammatory mediators like TNF α , MCP-1, and NGAL in donor kidneys treated with agents like recombinant human C1 inhibitor (rhC1INH) [162]. However, effectively addressing persistent issues such as delayed antibody-mediated rejection [163], managing underlying coagulation incompatibilities that can lead to TMA [164], and rigorously ensuring the absence of zoonotic infections [165,166] are all critical prerequisites before xenotransplantation can be considered a safe and viable therapeutic option for widespread use in immunocompetent human recipients.

5. Genetic Engineering of Porcine Donors

Recent breakthroughs in genome engineering technologies, particularly the development and refinement of CRISPR/Cas9 [7,167] and TALEN-mediated editing [168–170], have revolutionized the ability to create porcine donors with multiple, precise genetic modifications tailored for xenotransplantation. Notably, the generation of pigs engineered with a triple knockout (TKO) of the genes *GGTA1* (encoding α -1,3-galactosyltransferase) [171], *CMAH* (encoding cytidine monophosphate-N-acetylneuraminic acid hydroxylase)⁴³, and *B4GALNT2* (encoding β -1,4-N-acetyl-galactosaminyltransferase 2) [45,172] has been a cornerstone achievement. These three genes are responsible for synthesizing the primary carbohydrate xenoantigens recognized by preformed human antibodies. Consequently, TKO pigs exhibit significantly reduced susceptibility to HAR and antibody binding when their organs are exposed to human blood or transplanted into NHP models [173,174]. Moreover, building upon the TKO background, the addition (knock-in) of various human transgenes—such as those encoding complement regulators (*hCD46* [175], *hCD55*, *hCD59* [176,177]), coagulation modulators (*hTBM* [thrombomodulin] [178], *hEPCR*), and innate immune inhibitors (*hCD47* [179])—has further mitigated critical barriers like complement

activation, coagulation dysfunction, and macrophage-mediated phagocytosis, leading to markedly improved graft survival in preclinical models [76,77].

5.1. Rationale for Genetic Modifications

Xenotransplantation inherently confronts formidable immunological and physiological challenges. This is primarily because the human immune system is exquisitely evolved to recognize porcine tissues as foreign, mounting robust rejection responses that differ in intensity and mechanism from those seen in allotransplantation [22]. A pivotal factor driving the most immediate and severe form of rejection, HAR, is the presence of high titers of pre-formed natural antibodies in human serum. These antibodies primarily target the specific porcine carbohydrate antigen, galactose- α 1,3-galactose (α -Gal) epitope, which is abundantly expressed on porcine endothelial cells [54]. Binding of these antibodies (mostly IgM and IgG) to α -Gal rapidly activates the classical complement cascade, leading to endothelial cell damage, inflammation, thrombosis, and ultimately, rapid graft destruction, often within minutes to hours [180]. To mitigate this primary barrier, genetic engineering techniques, principally gene knockout, have been employed to create pigs lacking a functional *GGTA1* gene. Inactivation of *GGTA1* completely prevents the synthesis of the α -Gal epitope, thereby dramatically reducing the antigenicity of porcine tissues to humans and effectively preventing HAR [181].

Beyond HAR, other forms of rejection, collectively termed delayed xenograft rejection (DXR), present significant subsequent barriers. DXR involves a complex interplay of elicited antibody responses (against non-Gal antigens), further complement activation, endothelial cell activation, coagulation dysregulation, and infiltration by various immune cells [23,182]. Porcine cells naturally express their own set of complement regulatory proteins (CRPs) on their surface. However, these porcine CRPs are relatively ineffective at inhibiting the powerful activation of the human complement system [183]. To address this critical incompatibility, transgenic pigs have been developed to express key human CRPs directly on their cells. Commonly used human CRPs include *hCD46* (membrane cofactor protein), *hCD55* (decay-accelerating factor), and *hCD59* (protectin) [184]. Expression of these human proteins significantly enhances the regulation of human complement activation on the porcine endothelial surface, thereby reducing complement-mediated damage (like inflammation, cell lysis via MAC) and substantially improving graft survival in preclinical models.

Coagulation pathway incompatibilities also pose a critical

challenge, often leading to TMA [185,186]. Porcine tissues, when perfused with human blood, can trigger TMA due to several underlying differences in coagulation regulation between the species. For instance, as mentioned earlier, the high-affinity interaction between porcine vWF and human platelets drives excessive platelet activation and aggregation [187]. Furthermore, the porcine endothelium may not effectively activate human anticoagulant pathways (like the protein C system) or may express pro-coagulant molecules inappropriately. To counteract these pro-thrombotic tendencies, genetic modifications have introduced human anticoagulant transgenes, such as *hTM* (thrombomodulin) and *hEPCR* (endothelial protein C receptor), into pigs [188]. These proteins play crucial roles in promoting anticoagulation: hTM binds thrombin and shifts its activity towards activating protein C, a process greatly enhanced by hEPCR. Activated protein C then degrades key pro-coagulant factors Va and VIIIa, thus providing a natural brake on coagulation and reducing thrombotic complications [189].

Furthermore, innate immune cells, particularly macrophages and NK cells, contribute significantly to xenograft rejection through phagocytosis and direct cytotoxicity. Therefore, expressing human CD47 (*hCD47*) on porcine cells has been explored as a strategy to inhibit phagocytosis by human macrophages and potentially other myeloid cells [190]. *hCD47* functions by interacting with the inhibitory receptor SIRP α expressed on these human immune cells. This interaction delivers a potent inhibitory signal, often referred to as a "don't eat me" signal, which prevents the engulfment and destruction of the CD47-expressing porcine cells [191]. This genetic modification aims specifically to reduce innate immune responses directed against the graft endothelium and potentially other graft cells, thereby prolonging xenograft survival [192].

5.2. TKO Strategies

A foundational strategy in the genetic engineering of porcine donors involves the creation of triple-knockout (TKO) pigs. This is achieved through the simultaneous deletion or inactivation of three key glycosyltransferase genes: *GGT1*, *CMAH*, and *B4GALNT2* [193]. These genes encode the enzymes responsible for synthesizing the three major carbohydrate xenoantigens recognized by pre-formed human antibodies: α -Gal, Neu5Gc, and Sd(a), respectively [193]. The inactivation of *GGT1* eliminates α -1,3-galactosyltransferase activity, thereby completely abolishing the surface expression of the α -Gal epitope. This is critically important because α -Gal is recognized by a vast majority (>90%) of pre-existing human

IgM and IgG antibodies and serves as the principal trigger of HAR through classical complement activation and subsequent endothelial damage [87,194]. The *CMAH* gene encodes an enzyme that converts N-acetylneuraminic acid (Neu5Ac) into N-glycolylneuraminic acid (Neu5Gc). Neu5Gc is a sialic acid variant commonly found in most mammals but absent in humans due to an evolutionary inactivation of the *CMAH* gene. As a result, humans can develop anti-Neu5Gc antibodies (e.g., through diet) that bind to porcine tissues expressing Neu5Gc, contributing to antibody-mediated rejection [195]. Finally, the *B4GALNT2* gene produces the enzyme responsible for synthesizing the Sd(a) glycan epitope, another non-human carbohydrate structure that can be recognized by circulating human antibodies, potentially leading to complement activation and rejection [45].

Consequently, TKO pigs, lacking all three of these major xenoantigens, exhibit a markedly reduced binding of human natural antibodies (both IgM and IgG) to their cells and tissues *in vitro*. This translates to significantly increased resistance to HAR when their organs are perfused with human blood or transplanted into preclinical models *in vivo* [196]. For example, in kidney xenotransplantation studies using cynomolgus monkeys, grafts from TKO pigs that also expressed human transgenes for immune regulation (such as *hCD46* and *hTBM*) demonstrated survival extending beyond 180 days. Notably, this was often achieved without the need to pre-screen recipients for low anti-pig antibody titers, indicating the power of the TKO modification [197]. Histopathological examination of these surviving TKO grafts typically revealed minimal C4d deposition and an absence of the acute thrombotic microangiopathy often seen with non-TKO grafts during early post-transplant periods, further indicating improved immune compatibility and relative quiescence [198].

5.3. Human Transgene Additions

To further enhance cross-species compatibility beyond the elimination of key xenoantigens by TKO, porcine donors have been genetically modified to express multiple human transgenes. These transgenes are strategically chosen to target distinct immunological and coagulation pathways that remain incompatible between pigs and humans. One of the primary immunological barriers remaining after TKO is complement-mediated damage, which can contribute significantly to DXR [179,199,200]. As previously noted, native porcine complement regulatory proteins are ineffective at controlling the human complement cascade [201]. To overcome this critical limitation, human complement regulators like *hCD46*, decay-accelerating factor (*hCD55*), and protectin (*hCD59*)

have been successfully expressed in porcine endothelium via transgenesis. *hCD46* plays a key role by facilitating the Factor I-mediated enzymatic cleavage and inactivation of deposited C3b and C4b, thereby halting complement amplification early in the cascade [90]. *hCD55* functions by accelerating the decay (dissociation) of the C3 and C5 convertase enzyme complexes, thus reducing the generation of inflammatory anaphylatoxins (C3a, C5a) and downstream MAC formation [202]. *hCD59* acts at the final step of the cascade, directly inhibiting the polymerization of C9 and preventing the assembly of the lytic membrane attack complex (MAC), thereby directly protecting porcine cells from complement-mediated lysis [203].

Another major challenge in xenotransplantation is coagulation dysregulation, frequently resulting in the potentially catastrophic complication of TMA [204]. Porcine tissues tend to trigger human platelet activation and fibrin deposition due to inherent interspecies incompatibilities in critical anticoagulation pathways [205]. To counteract this pro-thrombotic state, porcine donors have been modified to express key human anticoagulant proteins, including *hTBM*, *hEPCR*, and more recently, *hTFPI* [64,141]. *hTBM* binds to thrombin, fundamentally changing its function from a procoagulant enzyme to an anticoagulant one by promoting the activation of protein C. Activated protein C then effectively degrades clotting factors Va and VIIIa, dampening further thrombin generation [206]. *hEPCR* significantly enhances this protein C activation process by presenting protein C to the thrombin–*hTBM* complex formed on the endothelial surface [207]. *hTFPI* (tissue factor pathway inhibitor) acts earlier in the coagulation cascade by inhibiting the TF–FVIIa complex, which is the key trigger of the extrinsic pathway, thereby dampening the initial burst of thrombin generation [64].

To further suppress innate immune responses, particularly phagocytosis by recipient macrophages and monocytes, the human *hCD47* transgene has been introduced into porcine donors. As described before, CD47 on the graft cells interacts with the SIRP α receptor on recipient myeloid cells, delivering an inhibitory signal that prevents the engulfment of these xenogeneic cells [190]. In preclinical models of kidney and heart xenotransplantation, expression of *hCD47* has been associated with observably reduced macrophage infiltration into the graft and consequently prolonged graft survival [124]. Additionally, the expression of *hCD39*—a human ectonucleotidase that hydrolyzes extracellular ATP and ADP released from activated platelets or damaged cells—has been employed. By converting these pro-thrombotic and pro-inflammatory nucleotides into AMP, *hCD39* helps to limit platelet activation and aggregation, further mitigating vascular

injury and inflammation within the xenograft microenvironment [208].

More recently, strategies targeting adaptive immune responses, specifically T cell and NK cell interactions, are being explored through transgene expression. Immune checkpoint regulators, including human leukocyte antigen E (*HLA-E*) and programmed death-ligand 1 (*PD-L1*), are currently under active investigation for their potential to inhibit NK cell and cytotoxic T lymphocyte (CTL) responses against the xenograft [209,210]. *HLA-E* expressed on porcine cells can engage the inhibitory receptor NKG2A found on human NK cells and some T cells. This interaction aims to prevent NK cell activation that might otherwise occur due to the "missing-self" signal resulting from the lack of appropriate human MHC class I molecules on porcine cells [211,212]. *PD-L1* expressed on graft cells can bind to the PD-1 receptor found on activated T cells (and some other immune cells). This binding delivers an inhibitory signal that suppresses T cell receptor-driven proliferation and cytokine production, potentially inducing T cell exhaustion or anergy [213,214]. Early results suggest that porcine cells engineered to express these molecules exhibit reduced susceptibility to lymphocyte-mediated cytotoxicity *in vitro* and in humanized mouse models. However, demonstrating clear efficacy and understanding the long-term impact of these immunomodulatory transgenes in large animal NHP models remains an area of ongoing evaluation [215–218].

Collectively, the coordinated expression of multiple human transgenes targeting these diverse pathways—complement, coagulation, innate immunity, and adaptive immunity—has yielded significant improvements in graft survival and functional outcomes in NHP studies. For example, kidney xenografts derived from TKO pigs co-expressing a suite of human transgenes including *hCD46*, *hCD55*, *hCD59*, *hTBM*, *hEPCR*, *hCD39*, and *hCD47* have achieved survival durations exceeding 6 months in cynomolgus monkeys, particularly when combined with anti-CD40-based immunosuppression [219,220]. Importantly, histological analysis of these long-surviving, highly modified grafts typically shows minimal C4d deposition, reduced infiltration by macrophages and platelets, and well-preserved renal architecture. These findings underscore the mechanistic efficacy of employing targeted, multi-transgenic strategies to successfully overcome many of the critical immunologic and physiologic barriers inherent to xenotransplantation [178]. However, it should be noted that overexpression of human transgenes, such as *hCD47* or *hTFPI*, may potentially elicit immune responses in recipients during long-term follow-up.

5.4. PERV Inactivation and Virological Safety

A unique safety concern specific to xenotransplantation is the potential transmission of porcine endogenous retroviruses (PERVs). PERVs are retroviral sequences that became integrated into the porcine germline DNA millions of years ago and are therefore present in the genome of all pigs [221,222]. While many PERV sequences are inactive, some retain the ability to produce viral particles. Studies have demonstrated that certain PERVs (particularly PERV-A, -B, and the recombinant -A/C) can indeed infect human cells *in vitro*, and may even be capable of transferring between human cells after initial infection [223,224]. This raises concerns about the theoretical risk of establishing a productive zoonotic infection in human recipients following clinical xenotransplantation.

To proactively address this potential virological risk, researchers have successfully employed CRISPR-Cas9 gene-editing technology to permanently inactivate PERV sequences within the porcine genome [7]. By designing guide RNAs that target highly conserved regions within the PERV *pol* gene, which encodes the essential reverse transcriptase enzyme required for viral replication, researchers were able to achieve simultaneous disruption of essentially all functional PERV copies throughout the genome in porcine kidney epithelial cells grown *in vitro* [225].

Subsequently, these PERV-inactivated cells were used as nuclear donors in the process of somatic cell nuclear transfer (SCNT, or cloning) to produce live cloned piglets [226]. Analysis of these resulting piglets demonstrated complete and stable inactivation of PERVs at both the genomic DNA level and the mRNA transcript level. Crucially, there was no detectable evidence of PERV particle production or reinfection from the surrogate mother pigs that carried the cloned embryos [227,228]. This groundbreaking achievement underscores the remarkable potential of CRISPR-Cas9-mediated PERV inactivation as a powerful tool to enhance the virological safety profile of porcine donor organs intended for xenotransplantation [229].

Building on this proof-of-concept, biotechnology companies focused on xenotransplantation, such as eGenesis, have incorporated PERV inactivation into their development pipelines. They have successfully developed porcine donor lines that harbor not only complete PERV inactivation but also extensive additional genetic edits, including multiple human transgenes aimed at improving immunological compatibility [230].

5.5. Donor Platforms and Manufacturing

Ensuring the safety and consistency of donor organs is

paramount for clinical xenotransplantation. Therefore, porcine donors currently being developed and utilized for preclinical studies aiming for clinical translation, as well as those used in the initial human transplants, are bred and maintained under rigorous designated pathogen-free (DPF) conditions. These conditions involve strict biosecurity protocols, frequent health monitoring, and exclusion of a long list of known porcine bacterial, viral, and parasitic pathogens in order to minimize the risk of transmitting conventional zoonotic infections (distinct from PERVs) to human recipients and provide a controlled microbial environment suitable for transplantation into immunocompromised patients [231,232].

Two leading biotechnology companies—United Therapeutics (through its subsidiary Revivicor) and eGenesis—have emerged at the forefront, developing highly specialized genetically engineered porcine platforms tailored specifically for clinical xenotransplantation applications [233]. Revivicor notably provided the 10-gene edited donor pig used in the first human cardiac xenotransplantation performed at the University of Maryland in January 2022 [34]. As detailed previously, this specific pig ('GalSafe® pig' platform base with added transgenes) underwent ten targeted genetic modifications. First, TKO modifications (*GGTA1*, *CMAH*, *B4GALNT2* KOs) eliminated the three primary carbohydrate xenoantigens to reduce hyperacute and delayed humoral rejection risk. Second, the pig was engineered to express six human transgenes: *hCD46*, *hCD55* (complement regulation), *hCD39* (platelet aggregation inhibition), *hTBM* and *hEPCR* (anticoagulation via protein C pathway enhancement), and *hCD47* (inhibition of macrophage phagocytosis via SIRPα interaction). Finally, the growth hormone receptor (*GHR*) gene was knocked out to prevent excessive organ growth (hypertrophy) after transplantation, aiming to maintain organ size within appropriate human physiological limits.

In parallel, eGenesis has advanced a distinct next-generation donor porcine platform. They utilize CRISPR-Cas9 technology, sometimes in combination with transposon-based systems, to introduce a potentially larger number of genomic edits—reportedly more than 40 in some research strains [234]. A key feature of the eGenesis platform is the integration of complete inactivation of all PERV loci, achieved by targeting conserved sequences in the *pol* gene [235], alongside the TKO modifications. Furthermore, their platform incorporates a comprehensive suite of human transgenes designed to address multiple immunological barriers simultaneously. These target not only complement and coagulation but also adaptive immunity (e.g., expressing *HLA-E*, *PD-L1*), inflammation, and cellular recognition pathways (e.g., *hCD47*, *hTFPI*). An

additional layer of sophistication involves engineering these donor pigs for precise control over transgene expression levels, potentially using tissue-specific promoters, to ensure appropriate and controlled expression in relevant vascular and parenchymal cell compartments within the organ [121,236].

Together, these advanced manufacturing platforms rely heavily on high-throughput SCNT for generating founder animals with the desired complex genotypes, coupled with rigorous virological screening and DPF breeding protocols to produce stable, pathogen-free herds suitable for organ procurement [226]. The ongoing convergence of multiplexed gene editing capabilities, vector safety engineering (for transgene insertion), and scalable breeding programs is crucially enabling the production of increasingly human-compatible porcine grafts. This progress is laying the essential groundwork for addressing the regulatory requirements and achieving the clinical scalability necessary for xenotransplantation to potentially become a therapeutic reality [17,237].

6. Immunosuppressive Strategies

The immunologic rejection of xenografts poses a more formidable challenge than that encountered in allotransplantation (human-to-human). This is because xenografts trigger responses from both the innate and adaptive arms of the immune system with greater intensity and involve unique rejection mechanisms [22,136,199]. Consequently, preventing rejection requires a tailored and often intensified immunosuppressive approach distinct from standard allotransplantation regimens.

At the molecular level, the earliest phase of rejection (HAR) is typically triggered by preformed natural antibodies, primarily IgM and IgG, binding to highly antigenic porcine carbohydrate structures like α -Gal expressed on the graft endothelium [112,238]. This antibody binding initiates the classical complement cascade, starting with C1q binding to the antibody-antigen complex. This leads to the sequential activation and cleavage of C4 and C2, forming the C3 convertase enzyme (C4b2a). This enzyme then cleaves abundant C3 into C3a (an inflammatory mediator) and C3b (which opsonizes the surface and amplifies the cascade) [239].

Subsequently, the formation of the C5 convertase leads to C5 cleavage into C5a (another potent inflammatory mediator) and C5b. C5b then initiates the assembly of the terminal membrane attack complex (MAC: C5b-9), which inserts into the endothelial cell membrane, causing cell lysis, inflammation, and thrombosis [240,241]. As discussed extensively, genetically modified pigs expressing human complement

regulatory proteins such as *hCD46*, *hCD55*, and *hCD59* have been developed precisely to interrupt this destructive sequence at multiple points [242]. Specifically, hCD46 acts as a cofactor for Factor I-mediated cleavage of C3b and C4b, halting amplification. hCD55 accelerates the decay of both C3 and C5 convertases, limiting their activity. hCD59 directly inhibits MAC formation by preventing C9 polymerization. Collectively, these transgenes significantly reduce complement-mediated cell lysis and inflammation [243].

Beyond humoral rejection and complement, T cell-mediated responses also play a critical role, particularly in delayed rejection phases. These responses can be initiated either through direct recognition, where recipient T cells recognize intact porcine MHC molecules (SLA in pigs) presented on donor antigen-presenting cells (APCs) within the graft, or through indirect recognition, where recipient APCs take up porcine antigens, process them, and present porcine peptides on their own human MHC molecules to recipient T cells [244]. The CD40–CD154 (CD40L) costimulatory pathway plays a central role in orchestrating these adaptive responses. CD154, expressed transiently on activated CD4⁺ T cells, binds to CD40, which is expressed on APCs (like B cells, macrophages, dendritic cells). This interaction is critical for licensing APCs to effectively prime T cells, and it provides essential 'help' signals for B cell activation, antibody isotype switching, and memory formation [245,246].

Early preclinical studies in NHP models demonstrated that blocking this pathway using monoclonal antibodies against CD154 could dramatically prolong xenograft survival. However, the clinical development of anti-CD154 antibodies was halted due to unexpected and serious thromboembolic events observed in human trials. These events were later attributed to the antibody binding to CD154 expressed on activated platelets, leading to platelet cross-linking and aggregation [247,248]. Consequently, the development of monoclonal antibodies targeting the *other* side of the interaction, CD40 (expressed on APCs but not significantly on resting platelets), has provided a potentially safer and highly effective alternative [76,249]. Antibodies like 2C10R4 (used in many NHP studies) and others currently in clinical development (like KPL-404) effectively block the CD40–CD154 interaction without directly activating platelets. Regimens based on anti-CD40 blockade have demonstrated remarkable success in NHP models, achieving long-term survival (e.g., over 400 days) of kidney and heart xenografts, often when administered intravenously at doses like 20 mg/kg every 1–2 weeks [84,250].

In addition to costimulation blockade, other

immunosuppressive agents are typically required, often in combination. Adjunctive agents such as belatacept—a fusion protein consisting of the extracellular domain of CTLA4 linked to an IgG Fc fragment—can provide additional T cell inhibition. Belatacept functions by binding to CD80 and CD86 on APCs with high affinity, thereby blocking their interaction with the CD28 receptor on T cells, which provides a crucial second signal for T cell activation [251,252]. Although perhaps less potent than anti-CD40 when used alone in the xenogeneic context, belatacept has been shown to enhance graft survival when combined with anti-CD40-based regimens in some NHP studies [253].

Standard induction immunosuppression protocols, administered peri-transplant, also often include potent lymphocyte-depleting agents like anti-thymocyte globulin (ATG). ATG contains polyclonal antibodies that recognize various T cell markers (like CD3) as well as markers on NK cells (like CD56), leading to their depletion from circulation via mechanisms like complement-dependent cytotoxicity and Fc receptor-mediated clearance [254,255]. High-dose corticosteroids, such as methylprednisolone (often given as a 500 mg IV bolus followed by tapering oral doses), are also standard during induction and for treating rejection. Corticosteroids exert broad anti-inflammatory effects by reducing the production of pro-inflammatory cytokines like IL-1 β , TNF- α , and IL-6, thereby dampening both innate and adaptive immune responses [126,131].

Maintenance immunosuppression typically relies on a combination of agents. Tacrolimus, a calcineurin inhibitor, remains a cornerstone. It acts by binding to the intracellular protein FKBP12, and this complex then inhibits the phosphatase activity of calcineurin. This inhibition prevents the dephosphorylation and subsequent nuclear translocation of the NFAT family of transcription factors, which are essential for transcribing key genes involved in T cell activation, most notably IL-2 [256]. Mycophenolate mofetil (MMF) is another standard maintenance drug. MMF inhibits the enzyme inosine monophosphate dehydrogenase (IMPDH), which is critical for the *de novo* pathway of guanine nucleotide synthesis. Because lymphocytes are highly dependent on this pathway for their proliferation, MMF effectively blocks both B and T lymphocyte expansion [132]. Together, these conventional agents suppress T cell clonal expansion and antibody production. However, due to long-term toxicities (especially nephrotoxicity associated with calcineurin inhibitors), there is increasing interest in developing maintenance regimens that

rely more heavily on costimulation blockers, potentially allowing for minimization or withdrawal of conventional agents [257].

Beyond pharmacological agents, cellular therapy strategies aimed at inducing tolerance or modulating the immune response are also under active investigation for xenotransplantation. One approach involves the infusion of donor-derived hematopoietic stem cells with the goal of inducing mixed hematopoietic chimerism. In this state, both porcine and human hematopoietic lineages coexist within the recipient's bone marrow and periphery. This coexistence can promote central immune tolerance by facilitating the deletion of newly developing porcine-reactive T cells within the thymus [258]. Another promising cellular approach involves the use of regulatory T cells (Tregs). Tregs, typically characterized by expression of the transcription factor FOXP3, play a crucial role in maintaining immune homeostasis by actively suppressing effector T cell responses. They exert their suppressive functions through various mechanisms, including the secretion of immunosuppressive cytokines like IL-10 and TGF- β , and by downregulating the expression of costimulatory molecules like CD80/CD86 on APCs via surface CTLA-4 [259,260]. In NHP xenotransplantation models, the co-infusion of autologous (recipient-derived) Tregs (e.g., 5–10 million cells/kg), particularly when combined with costimulation blockade, has shown potential in attenuating graft infiltration and improving graft survival [261]. Similarly, mesenchymal stromal cells (MSCs) are being explored for their immunomodulatory properties. MSCs can exert broad immunosuppressive effects by secreting various factors, including indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), and nitric oxide (NO). These mediators can suppress T cell proliferation, promote Treg development, and polarize macrophages towards an anti-inflammatory M2 phenotype [262].

Together, these multifaceted approaches—including advanced genetic engineering of the donor, pharmacological inhibition of complement and key immune pathways (especially costimulation blockade), and potentially cellular modulation strategies—form the basis of current and future protocols aimed at achieving long-term xenograft survival (Figure 3). The ultimate goal of integrating these approaches is not only to prolong graft survival but also to reduce the burden and toxicity of lifelong immunosuppression, and perhaps eventually, to induce a state of durable operational tolerance in immunocompetent human recipients [27].

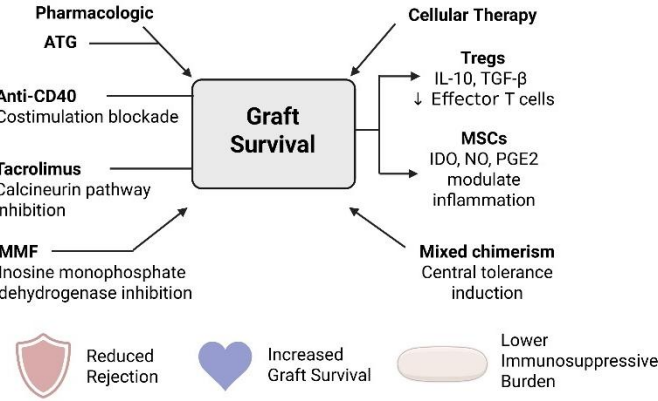


Figure 3. Comparison between allotransplantation and xenotransplantation.

This figure compares human-to-human allotransplantation with Porcine-to-human xenotransplantation. Allotransplantation is widely practiced with established immune compatibility but is limited by donor shortages. In contrast, xenotransplantation offers a potentially unlimited organ supply but faces major challenges including immune rejection, coagulation disorders, and zoonotic infection risks such as porcine endogenous retrovirus (PERV) and porcine cytomegalovirus (PCMV). The table summarizes key differences in donor availability, infection risk, and immunogenicity.

7. Summary and Future Directions

Organ transplantation remains the definitive treatment for end-stage organ failure, yet critical donor shortages severely limit allotransplantation. Xenotransplantation, particularly using genetically engineered pigs, offers a potential solution to this crisis but faces significant immunological, physiological, and virological hurdles before widespread clinical adoption is feasible (Figure 4).

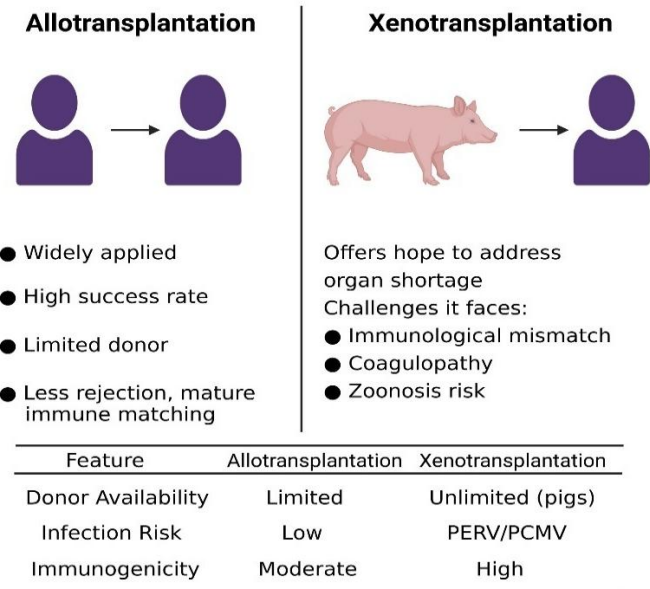


Figure 4. Strategies to enhance graft survival in xenotransplantation.

This figure summarizes key immunosuppressive and immunomodulatory strategies aimed at promoting graft survival. Pharmacologic agents include anti-thymocyte globulin (ATG), anti-CD40 antibody (costimulation blockade), tacrolimus (calcineurin pathway inhibition), and mycophenolate mofetil (MMF, inosine monophosphate dehydrogenase inhibition), which reduce rejection and improve graft longevity. Cellular therapies involve regulatory T cells (Tregs), which secrete interleukin-10 (IL-10) and transforming growth factor- β (TGF- β) to suppress effector T cells, and mesenchymal stem cells (MSCs), which produce immunomodulatory mediators including indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO), and prostaglandin E2 (PGE2). Mixed hematopoietic chimerism supports central immune tolerance. Together, these approaches aim to reduce immune rejection, prolong graft survival, and minimize long-term immunosuppressive burden.

Looking ahead, research must prioritize achieving consistent, long-term graft survival in preclinical models. This requires further optimizing genetic engineering strategies—integrating effective complement regulators, anti-phagocytic molecules, and coagulation modulators—with next-generation immunosuppression, especially costimulation blockade, potentially reducing reliance on toxic agents. Addressing organ-specific challenges is also crucial; for instance, overcoming the severe thrombocytopenia and coagulopathy in liver xenotransplantation necessitates targeted approaches like vWF gene humanization or novel perfusion techniques.

Concurrently, advancing tolerance-inducing strategies, such as mixed hematopoietic chimerism or regulatory T cell therapies, is essential to significantly reduce the burden of lifelong immunosuppression. Success also hinges on establishing large-scale, reproducible manufacturing of genetically stable, designated pathogen-free (DPF) donor pigs, a focus of ongoing commercial efforts.

Ultimately, the field must progress towards meticulously designed clinical trials in immunocompetent human recipients. These initial trials, prioritizing safety and efficacy, will be transformative in determining the viability of xenotransplantation as a clinical reality. The path forward demands synergistic advances in molecular engineering, immune monitoring, infectious disease control, and careful ethical and regulatory oversight (Figure 5). Continued multidisciplinary collaboration is essential to mitigate current limitations and realize the profound potential of xenotransplantation to provide sustainable, life-saving therapies for patients worldwide.

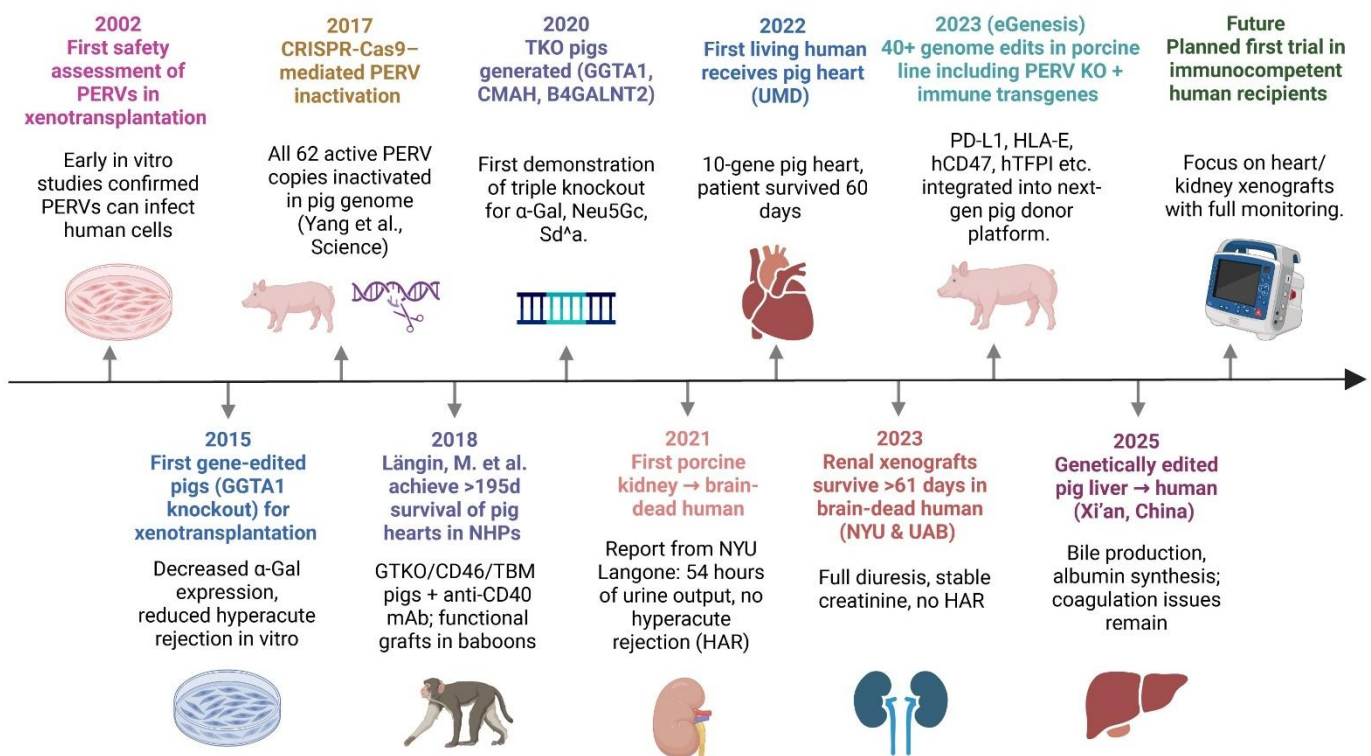


Figure 5. Timeline of key milestones in xenotransplantation development.

This timeline highlights major breakthroughs in xenotransplantation from early in vitro studies to recent clinical progress. Initial safety assessments of porcine endogenous retroviruses (PERVs) in 2002 led to CRISPR-Cas9-mediated PERV inactivation in 2017. Gene editing advances include triple-knockout (TKO) Porcines and integration of human immune regulatory genes. Landmark events include long-term Porcine heart survival in non-human primates (2018), the first Porcine heart and kidney xenografts in brain-dead humans (2021–2023), and genetically edited Porcine liver xenotransplantation (2025). Future directions focus on trials in immunocompetent human recipients with comprehensive immune and coagulation monitoring.

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