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Europeans and Xenotransplantation

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Editorial: Europeans and xenotransplantation

Emanuele Cozzi[1]* and David K. C. Cooper[2]

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Editorial on the Special Issue
Europeans and xenotransplantation

Recent developments in the field of xenotransplantation have attracted considerable attention and constitute a major turning point for this scientific discipline. These recent investigations illustrate the major progress that has taken place in the last decade. In particular, advances in genetic-engineering techniques have allowed the generation of donor pigs whose organs are increasingly compatible with humans from both an immunological and physiological perspective. In addition, the use of novel immunosuppressants has contributed to major advances in preclinical studies in which life-supporting pig organs have been able to sustain the lives of many nonhuman primates for more than a year, and even for 5 years in one case.

Such impressive results have opened the way for the first xenotransplantation procedures in human patients. The initial reports on the clinical results obtained to date may be considered by many to be perhaps mixed but they provide considerable encouragement. They have drawn attention to several aspects that require further investigation and, although the road remains an uphill climb, we are encouraged that further progress will likely be made in the near future. Indeed, as never before, xenotransplantation is now perceived as a probable solution to the world-wide shortage of human organs.

When we were invited by the Editor-in-Chief of *Transplant International,* to act as Guest Editors for a special issue of the journal on the latest developments in the field of xenotransplantation, we realized that many review articles had been published during the previous 24 months on various aspects of xenotransplantation. How could we throw some new light on the topic or address it from a novel perspective? As *Transplant International* is the official journal of the European Society for Organ Transplantation, we decided to concentrate attention on research carried out largely by authors who were born or educated or are currently living in Europe. In addition, we decided to invite a handful of clinical experts at the forefront of allotransplantation in Europe (with or without a special interest in xenotransplantation) to share with us their opinions on the current status of xenotransplantation and how they anticipate it will be introduced clinically.

Although much of the research into xenotransplantation has been carried out at centres in the USA, in many cases the work has actually involved a large number of Europeans who had either relocated permanently or temporarily to the USA. We therefore began by summarizing the many contributions of Europeans to (i) pig organ xenotransplantation

(Iwanczyk et al.) and (ii) pig islet and cell xenotransplantation (Bottino et al.). Much of this work was carried out by young surgeons or physicians gaining experience in research before returning to their home countries in Europe, where several are now leaders in the field of transplantation.

Of those who remained in the USA, islet xenotransplantation, in particular, has benefitted by the sustained efforts of Europeans such as Bernhard Hering (Germany) and Rita Bottino (Italy). More recently, Pierre Gianello and his colleague, Nizar Mourad, in Belgium, working with Cesare Galli (Italy), have introduced a novel genetic modification by which pig pancreatic islets may produce much more insulin than those from wild-type pigs (Mourad and Gianello). As pigs need and produce less insulin than humans, these specific gene-edits may enable normoglycemia to be achieved in a diabetic recipient by the transplantation of fewer pig islets.

There have been several contributions by Europeans on both sides of the Atlantic in such areas as (i) the genetic engineering of the organ-source pigs, e.g., by the late David White (UK), Eckhard Wolf (Germany), and Cesare Galli (Galli; Ali et al.), (ii) the development of an effective immunosuppressive regimen, e.g., by Leo Buhler (Switzerland), who demonstrated the efficacy of CD40/CD154 co-stimulation blockade in xenotransplantation (Sanatkar et al.), (iii) pig liver xenotransplantation, e.g., Burcin Ekser (Turkey and Italy) (Sucu et al.), and (iv) the potential problems of infectious complications in xenotransplantation, in which several Europeans have become acknowledged leaders in the field, e.g., Joachim Denner (Germany), Linda Scobie (UK), and Nicolas Mueller (Switzerland) (Mueller and Scobie; Denner). In the important realm of basic science research, Joerg Seebach (Switzerland) (Galdina et al.) and Robert Rieben (Switzerland) (Gultom and Rieben) have both contributed significantly over many years.

Several Europeans (particularly from Erasmus University in The Netherlands) contributed to progress in pig heart xenotransplantation, including an assessment of the first α1,3-galactosyltransferase gene-knockout (GTKO) pigs (Iwanczyk et al.). Subsequently, the Munich group, founded and led by Bruno Reichart (Germany), played a leading role in establishing successful orthotopic pig heart transplantation in nonhuman primates, which had been elusive until then (Längin et al.). Progress in pig kidney and lung xenotransplantation has also progressed through the work of Europeans in the USA and in Europe (Iwanczyk et al.; Le Bas-Bernardet et al.; Takemoto et al.). The importance of the pig-to-nonhuman primate model, in which most of the advances we have seen over the past 40 years were developed, has been emphasized, particularly when compared with studies in human decedents (Cooper et al.). Commentaries on pig organ xenotransplantation have been contributed to this collection of papers by leaders in the field of organ allotransplantation (Karadagi and Oniscu; Böhmig et al.; Piemonti et al.). Finally, this special issue also includes a paper illustrating aspects of regulation of xenotransplantation in Europe (Tönjes).

We hope you will find the collected papers informative with regard to the important, and often overlooked, role that Europeans have played in the research that has enabled clinical trials of xenotransplantation to be initiated.

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REVIEW
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Contributions of Europeans to Xenotransplantation Research: 1. Pig Organ Xenotransplantation

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Xenotransplantation has a rich history, marked by European pioneers who laid the groundwork for many breakthroughs in the field. Pig organ xenotransplantation offers a solution to the global shortage of deceased human donor organs, whilst allowing the modification of the donor graft itself. The field has continued to garner interest, particularly with the recent advent of simpler and faster genetic-engineering technologies. This review highlights the contributions of European researchers to xenotransplantation, spanning pig kidney, heart, liver, and lung transplantation. Research has focused on (i) identifying and deleting key xenoantigens and modifying the source pig by expression of human "protective" proteins and (ii) testing novel immunosuppressive regimens. These contributions have played key roles in advancing xenotransplantation from the laboratory to early clinical experiments. Europeans have also addressed the potential risks of xenozoonotic infections and the regulatory challenges. The research endeavours of groups in Europe are summarized. Several European researchers moved either permanently or temporarily to US institutions, and their insight and innovations are also highlighted. While we aim to recognize the significant contributions of European physicians and scientists in this article, it is not an exhaustive list of all those who have influenced the field.

Keywords: Europeans, organs, pig, USA, xenotransplantation

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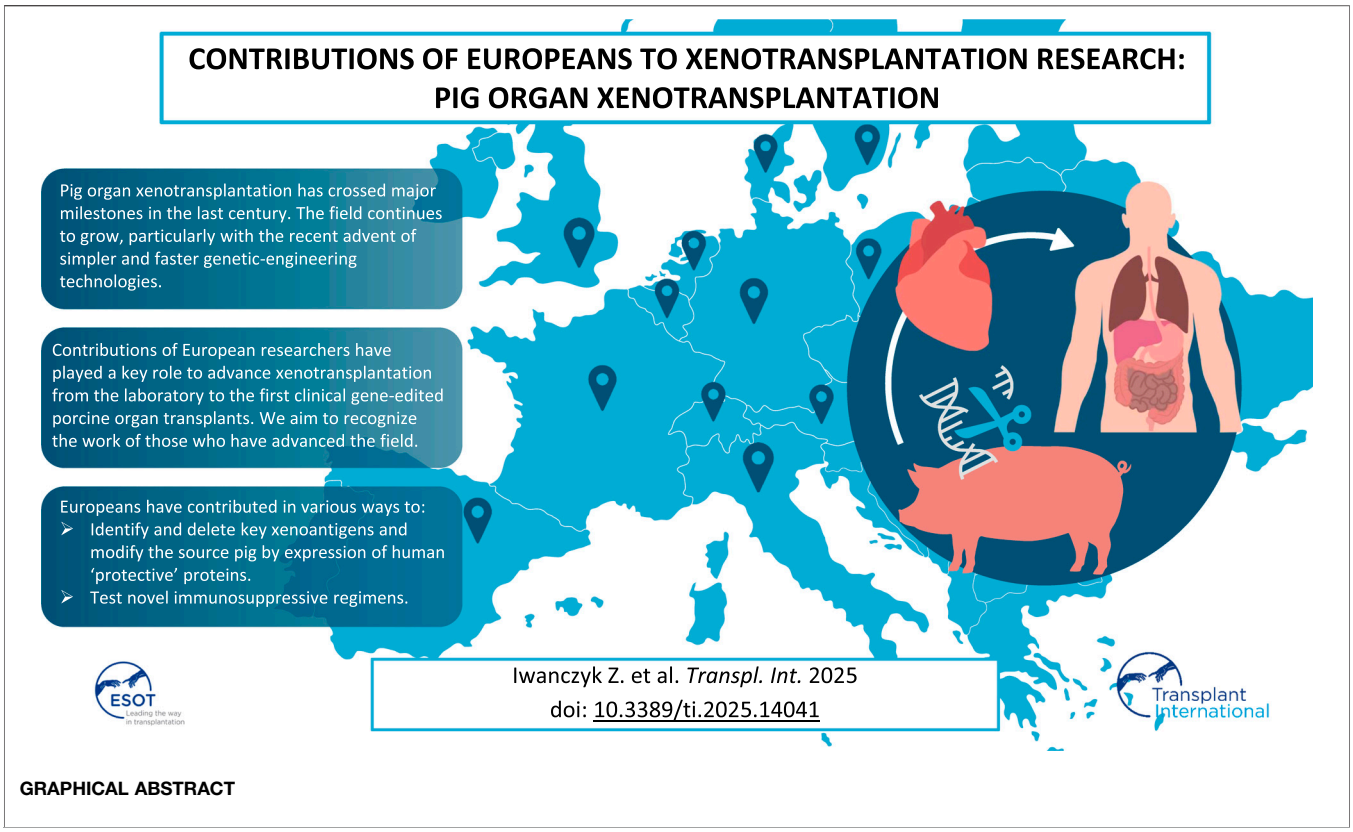
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INTRODUCTION

Europeans played a pioneering role in the late 19th and early 20th centuries by transplanting organs from various animals into human recipients - with very poor results [1, 2]. The work of the French experimental surgeon, Alexis Carrel, needs to be highlighted because he and his American colleague, Charles Guthrie, introduced a more reliable technique of anastomosing blood vessels, without which the transplantation of organs would have proved difficult. For this contribution, Carrel was awarded the Nobel Prize for Physiology or Medicine in 1912.

There was then a lull in advances in transplantation until the Ukrainian, Yu Yu Voronoy, carried out a small series of clinical kidney *allo*transplants over several years beginning in the 1930s. However, he did not explore the use of animals as potential sources of organs. Activity in

Abbreviations: Gal, galactose-α1,3-galactose; GTKO, α1,3-galactosyltransferase gene-knockout; hDAF, human decay-accelerating factor; NHP, nonhuman primate.



GRAPHICAL ABSTRACT

xenotransplantation began again with the work of the American, Keith Reemtsma, who transplanted chimpanzee kidneys into six patients in 1963, one of whom lived for 9 months. This was closely followed by several other American and European surgeons who transplanted kidneys from nonhuman-primates (NHPs) and by James Hardy (USA) and his colleagues who carried out the world's first clinical heart transplant, again from a chimpanzee. American surgeon, Tom Starzl, soon followed by transplanting livers from NHPs into human recipients, again with little success.

Only one surgeon during this era, the Frenchman Rene Küss, a very early pioneer in kidney allotransplantation, transplanted a kidney from a pig; he was shocked by the rapid speed of rejection - that occurred within minutes.

It was not until the mid-1980s that serious attention turned from NHPs to pigs as the source of organs. There were several logistical reasons for this redirection of attention (**Table 1**). However, a few years earlier, one European surgeon, Roy Calne, had explored pig liver transplantation in NHP recipients.

ROY CALNE (UNITED KINGDOM) – PIG LIVER TRANSPLANTS IN NHPS

The late Roy Calne, renowned for his work in clinical allotransplantation, left a legacy of firsts within his field [3]. He is most remembered for the introduction of immunosuppressive therapy, notably including azathioprine, cyclosporine, rapamycin, and alemtuzumab (Campath). Working at the University of Cambridge, he also carried out the first liver transplant in Europe in 1968, the world's first combined liver-heart-lung transplant in 1987 (with cardiothoracic surgeon John Wallwork), and the first intestinal transplant in the United Kingdom. However, Calne also contributed to early work in xenotransplantation by carrying out a small series of pig liver transplants in NHP recipients in the late 1960s [4, 5]. The results were again disappointing.

Some years later, Calne suggested the terms "concordant" and "discordant" xenotransplants to distinguish between those rejected relatively slowly by a cellular mechanism (similar to allotransplantation) and those rejected hyperacutely by an antibody-dependent complement-mediated mechanism (similar to that seen in ABO-incompatibility or in HLA-sensitized recipients) [6].

THE NEXT STEP

In the early 1980s, one of the present authors (David Cooper [United Kingdom]), while working at the University of Cape Town, carried out a study on xenotransplantation between different species of NHPs (a concordant model) [7]. In 1985, he and another European, Gerfried Lexer [Austria], established what has become a standard experimental model in xenotransplantation – the pig-to-NHP heterotopic heart transplant model (a discordant model), in which the heart is

TABLE 1 | The advantages and disadvantages of the pig as a potential source of organs and cells for humans.

Availability	Unlimited, whenever required
Breeding potential	Good
Period to reproductive maturity	4–8 months
Length of pregnancy	114 ± 2 days
Number of offspring	5–12
Growth	Rapid (adult human size within 6 months)[a]
Size of organs for all ages of humans	Adequate
Anatomical similarity to humans	Close
Physiological similarity to humans	Close
Relationship of immune system to humans	Distant
Knowledge of tissue typing	Considerable (in selected herds)
Blood type compatibility with humans	ABO-blood type compatibility can be assured [All pigs will be of type O (non-A)]
Experience with genetic engineering	Considerable
Risk of transfer of infection (xenozoonosis)	Low
Availability of designated pathogen-free pigs	Yes
Cost of maintenance	Under the biosecure designated pathogen-free conditions required by the national regulatory authorities the costs will be significant
Public opinion	Generally supportive considering that, in the US alone, more than 100 million pigs are slaughtered annually for feeding purposes only

[a]*Various miniature pigs reach a maximum weight of 10%–50% of the weight of domestic pigs.*

anastomosed to vessels in the recipient's abdomen or neck but is not contributing to the support of the circulation [8, 9]. They confirmed that rapid rejection of a pig graft (hyperacute rejection) occurred and was a result of the binding of human serum anti-pig antibodies to the graft and the activation of the complement cascade. Treatment with the available immunosuppressive drugs had little or no effect in delaying rejection [9].

They introduced the concept of perfusing one organ from the pig, e.g., the kidney, to adsorb anti-pig antibodies from the potential recipient's blood before transplanting another organ, e.g., the heart, from the same pig.

GUY ALEXANDRE (BELGIUM) – PIG KIDNEY TRANSPLANTS IN BABOONS

The late Guy Alexandre (Catholic University of Louvain) took it one step further by transplanting a life-supporting pig kidney intra-abdominally and removing the recipient's native kidneys [10]. Like Calne, Alexandre was an early contributor to organ allotransplantation [11]. In 1963, he was the first to suggest and implement the use of kidneys from brain-dead donors (i.e., before circulatory death), a notion that was very controversial at the time. He went on to introduce methods to safely transplant ABO-incompatible kidneys by prior removal of anti-AB antibodies by plasmapheresis. These two major innovations, the use of organs from brain-dead donors and ABO-incompatible organ transplantation, have done a great deal to increase the donor pool of allografts and, in our opinion, Alexandre did not receive sufficient recognition for these contributions. For example, he was not awarded the prestigious Medawar Prize of The Transplantation Society.

However, in addition, he was a very early contributor to research into xenotransplantation. Using his experience of clinical ABO-incompatible allotransplantation, he utilized plasmapheresis to remove anti-pig antibodies from the NHP recipient before pig kidney transplantation. He achieved the longest survival time of baboons with a pig kidney at the time (22 days) in 1989 [10]. Unfortunately, although 'accommodation' developed after many ABO-incompatible allotransplants (in which the return of anti-AB antibodies was *not* followed by rejection), it did not occur after xenotransplantation. Although xenograft survival was short compared with that which we achieve today, Alexandre's work undoubtedly played a role in advancing xenotransplantation.

THE IMUTRAN (UNITED KINGDOM) GROUP

In the mid-1980s after the introduction of cyclosporine as an immunosuppressant, the number of patients waiting for a transplant significantly increased world-wide and it became apparent that there was a growing need for an alternative source of organs for patients with terminal organ failure.

In this context, xenotransplantation was viewed by some as one of the possible approaches to explore, although the immunological barriers to successful xenotransplantation, in particular, hyperacute rejection, were major obstacles to overcome. Scientists at that time estimated that there were two approaches to overcome this immunological barrier. The first consisted in the development of new molecules or immunotherapies to enable the recipients to accept the xenograft. The second was to "manipulate" the pig organ to render it more compatible with the recipient.

Among those greatly interested in finding a solution to the shortage of human organs and render xenotransplantation a viable option was University of Cambridge transplant immunologist, David White, who was well-known in the transplantation community for his contributions, with Roy Calne, to the development and clinical introduction of

cyclosporine. White immediately understood that working on the donor (as opposed to the recipient) was the road to pursue. He used the largely unrecognized fact (at that time) that complement-regulatory proteins are species-specific (and prevent autologous complement from injuring native cells), to explore whether the transgenic expression of a human complement-regulatory protein in pigs would protect the pig vascular endothelial cells from injury by human complement [12]. (Augustin Dalmasso in the USA had the same idea [13].).

In the early 1990s, White, together with his surgical colleague, John Wallwork, and a local businessman, founded Imutran, a biotech company based in Cambridge whose key objective was to pave the way to clinical xenotransplantation [12, 14, 15]. The researchers were mainly British but with a number of foreign team members. Among the most important were molecular biologists Nikos Yannoutsos (Greece) and Gillian Langford (United Kingdom), and Richard Lancaster (United Kingdom) who had expertise in the generation of transgenic pigs by pronuclear microinjection of fertilized oocytes with purified DNA [16–18].

The initial *in vitro* experiments conducted by White's team (that included one of the present authors [Emanuele Cozzi, Italy]), and by other groups in the United States, showed that murine cells expressing a human complement inhibitor, such as human decay-accelerating factor (hDAF), were resistant to human complement injury. Such experiments led to the generation of transgenic mice expressing hDAF in various tissues whose cells were resistant to human serum complement-mediated lysis [19]. These convincing results suggested that an appropriate gene-edited pig might possibly represent an unlimited source of organs for clinical transplantation and encouraged Imutran and other companies (such as Nextran and Alexion, both in the United States) to invest considerable resources in this specific area of research. Indeed, the competition at that time was severe. These companies received funding from "big pharma" companies already involved in the healthcare sector. In particular, Imutran received funding from Sandoz (that soon merged with Ciba-Geigy to become Novartis) while Nextran was supported by Baxter.

Thanks to the support provided by Sandoz/Novartis, Imutran could strengthen its infrastructure, reinforce its manpower and logistics, and initiate an intense research activity aimed at genetic engineering pigs as a possible source of organs for clinical purposes. To this end, Imutran appointed a team of molecular biologists with expertise in gene-editing of cells and made the necessary investments to enable the opening and maintenance of facilities necessary for the generation and breeding of genetically-engineered pigs. The results were promising with the successful birth of the first hDAF gene-edited pig litter during the final days of 1992, an achievement that opened the way to fundamental proof-of-concept studies.

As a first step, *ex-vivo* perfusion studies demonstrated that organs from hDAF transgenic pigs were indeed resistant to human complement-mediated injury. Subsequently, hDAF pig hearts were transplanted heterotopically into non-immunosuppressed NHPs and proved to be resistant to hyperacute rejection, with a median survival of 5.1 days. In immunosuppressed NHPs the hearts survived for up to 62 days, an accomplishment that enabled the initiation of life-supporting studies in NHPs.

Such encouraging results boosted the visibility of Imutran and encouraged Novartis to purchase the company. However, they soon also attracted the attention of 'animal rights' activists in the United Kingdom which was one factor - together with concern of the medicolegal risks of the transfer of porcine endogenous retroviruses (PERVs) to a patient with a pig graft - that eventually persuaded Novartis to shut down Imutran and transfer its know-how to a newly created Boston US-based company, Immerge.

Before its demise, Imutran contributed much to advance pig heart and kidney xenotransplantation, and also carried out pig liver transplantation with a Spanish team [20]. It also generously provided some of its hDAF pigs to centers in the United States, which did much to stimulate the entire field of xenotransplantation research. In summary, Imutran's work provided the first step towards the use of gene-edited pigs as sources of organs for clinical transplantation.

EARLY WORK IN DENMARK

During the 1980s and 1990s, Ejvind Kemp and his colleagues in Odense and Copenhagen were very active in various fields of xenotransplantation research, ranging from rodent studies [21] to the histopathology of rejection [22] to large animal experiments [23]. Ejvind Kemp's great interest in this new field of research is exemplified by his authorship of a book on xenotransplantation as early as 1978 [24].

GLYCOBIOLOGY AND CLINICAL EXPERIMENTS IN SWEDEN

Swedish groups proved very active in the early days of xenotransplantation research, especially in islet transplantation (see Bottino et al, this issue) in part because of excellent collaboration between their major medical universities. Swedish surgeon/scientist, Michael Breimer (initially with Bo Samuelsson) at Gothenborg University, was active in the field of glycobiology in relation to pig organ xenotransplantation. He characterized the structure and tissue distribution of carbohydrate xenoantigens from several organs and tissues from various wild-type and gene edited pigs [25–27].

In addition, after discussion with Ken Welsh, David Taube, and others in the United Kingdom [28], the Swedish team carried out two bold experiments in 1996 [27, 29, 30]. With the consent and cooperation of two patients undergoing regular dialysis, they inserted a wild-type pig kidney into the circuit so that the kidney was perfused with the patient's blood. Previous plasmapheresis had been carried out to deplete anti-pig antibodies and no immunosuppressive therapy was administered. In one patient, the kidney survived for 65 min before being rejected. The second patient developed hemodynamic shock within 15 min, presumed to be associated with release of cytokine and complement factors, necessitating termination of the experiment.

TABLE 2 | Carbohydrate xenoantigens that have been deleted in genetically-engineered pigs.

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

Hakan Widner and his colleagues at the University of Lund were very active in the field of embryonic fetal pig brain tissue transplantation to cure neurodegenerative conditions [31].

THE WORK OF EUROPEANS IN THE UNITED STATES

David Cooper relocated to the United States where he worked at various centers and collaborated with several European scientists – some temporarily or permanently resident in the USA and others based in Europe. He participated in the mentoring of several young physicians and surgeons who went to the USA to gain experience in xenotransplantation. Several of these young men and women made major contributions and some have since become leaders in the field of transplantation.

Oklahoma Transplantation Institute and Oklahoma Medical Research Foundation (OMRF)

Initially based in Oklahoma City, Cooper's group collaborated with a small Canadian biotech company (Chembiomed, based in Edmonton) to identify the major carbohydrate pig antigen against which humans (and NHPs) have natural (preformed) antibodies that initiate hyperacute rejection. Based on what was known about ABO blood group antigens (which are oligosaccharides), the Oklahoma team reasoned that anti-pig antibodies may also be directed to pig carbohydrate antigens. This proved correct. A key and very important member of the Oklahoma team, Eugene Koren, an OMRF scientist from Croatia, was responsible for isolating the antibodies that bind to pig vascular endothelial cells. These were then tested in Edmonton against a panel of synthetic oligosaccharides and found to bind to galactose-α1,3-galactose (Gal) (**Table 2**) [32, 33]. This was a major step forward and confirmed an observation (hitherto unknown to the Oklahoma team) made earlier by Uri Galili (from Israel).

Their original plan was to deplete the human serum of anti-Gal antibodies by immunoadsorption on columns of synthetic Gal glycans but, during discussion with another close collaborator, Paris-based immuno-geneticist Rafael Oriol, it was determined that the ultimate solution would be to delete expression of Gal in the pig [34]. The technology to do this had not yet been developed and so it was more than a decade before α1,3-galactosyltransferase gene-knockout (GTKO) pigs became available [35, 36].

In the meantime, the team developed methods of immunoadsorption to deplete baboons of anti-Gal antibodies. Marek Niekrasz (Poland) was one of the veterinary surgeons who participated fully in these challenging endeavors. Anti-Gal antibodies were successfully removed from the serum but, despite immunosuppressive therapy, returned within a few days, at which time rejection occurred. There was some collaboration with Robert Rieben's team (Switzerland) [37], and with a group in the United Kingdom (David Taube, Tom Cairns, Ken Welsh) who were also pursuing this approach, and members of this group visited Oklahoma City to participate in these experiments [28, 38].

Gene Koren led a small and productive subgroup of the team (that at times included several other Croatians) to carry out numerous *in vitro* studies that identified IgM, IgG, IgA, and IgE anti-Gal antibodies [39–42]. Back in Paris, Rafael Oriol investigated numerous animal species for expression of Gal [43, 44].

Identification of N-Glycolylneuraminic Acid as a Second Pig Carbohydrate Xenoantigen

While these studies were taking place in the USA, a French group identified N-glycolylneuraminic acid (Neu5Gc) as a second pig xenoantigen (**Table 2**) [45], but this observation was not pursued at that time. It was not until Alex Zhu confirmed this finding in 2002 that interest in Neu5Gc as a xenoantigen began to expand [46].

Massachusetts General Hospital (MGH)/ Harvard Medical School

After approximately 9 years, the Oklahoma team split up, and Cooper accepted an appointment at the Transplantation Biology Research Center (headed by David Sachs) at the MGH. With David Sachs, they made a sustained effort to induce a state of immunological tolerance to pig organs in NHPs, but this proved too great a challenge with the therapies available at the time. The team explored the effect of various immunosuppressive drugs on the "rebound" of anti-pig antibodies after immunoadsorption [47]. Key European participants included Denis Lambrigts (Belgium) and his wife Denise van Calster (Belgium), who arrived at a critical time and did much to improve the productivity of the xenotransplantation program, Leo Buhler (Switzerland), Ian Alwayn and Frank Dor (both from surgeon Jan Ijzermans' group at Erasmus University in Rotterdam in Netherlands). Frank Dor's major interest at MGH was the induction of tolerance by spleen allotransplantation, but he was involved in all of the xenotransplantation work.

It was then that another major observation was made. In their studies of pig hematopoietic progenitor cell (HPC) transplantation as a potential means of inducing tolerance to pig xenoantigens, Leo Buhler observed that, despite cyclosporine-based immunosuppressive therapy, the infusion of pig HPCs was followed by sensitization, resulting in a great increase in the titers

of anti-pig IgM and, particularly, IgG [48]. Knowing of the introduction of new agents that caused blockade of the T cell co-stimulation pathway in allotransplantation, Buhler found that treatment with an anti-CD154 mAb (rather than cyclosporine) prevented sensitization [48]. This proved to be a major advance in xenotransplantation and, since that time (2000), all truly successful immunosuppressive regimens administered to NHPs with pig organ or cell grafts have been based on CD40/CD154 co-stimulation pathway blockade.

Coagulation dysfunction had been predicted by several physicians interested in xenotransplantation research, including Simon Robson [United Kingdom and South Africa, by then based in Boston in the USA, where he was joined by a series of European research fellows, e.g., Matz Schmelze (Germany), Jan Schulte am Esch (Germany), Christoph Kopp (Austria), and Daniel Candinas (Switzerland)]. However, it was at the MGH that several fundamental observations were made from the pig-to-NHP studies progressing at that time. Key observations were made by Thomas Kozlowski (Poland), Frank Ierino (Australia), and Leo Bühler who independently observed that rejection of a pig organ was accompanied by a thrombotic microangiopathy in the graft and a disseminated coagulopathy in the recipient [49, 50]. Ian Alwayn (Netherlands), working partly with Simon Robson, made several significant contributions to our understanding of these phenomena [51–54].

When GTKO pigs were born at Immerge in Boston [36], the team progressed to the transplantation of their organs into NHPs, using an anti-CD154mAb-based immunosuppressive regimen (reviewed by Sanatkar et al in this issue). Hendrik-Jan "Henk" Schuurman (Netherlands), then the vice president for research at Immerge, played a leading role in the production of these pigs. Heterotopic heart transplantation was associated with longer function than reported previously, with maximal survival of 6 months [55]. Maximal survival of baboons with a life-supporting GTKO pig kidney graft was approximately 3 months [56].

Christoph Knosalla (Germany) and Bernd Gollackner (Austria) joined the team. Christoph continued the GTKO pig heart transplant studies but faced challenging problems with coagulation dysfunction [57, 58]. Bernd carried out some valuable *in vitro* studies with Simon Robson [59, 60], Nicolas Mueller (Switzerland) and Jay Fishman [61–63].

Despite the success that Immerge had achieved, Novartis withdrew its financial support, just as it had withdrawn support for Imutran a few years earlier. When Immerge was forced to close, the core of the xenotransplant research team was invited (by Tom Starz) to relocate to Pittsburgh, where the University of Pittsburgh Medical Center (UPMC, the company that owned several hospitals in the region) had recently purchased the small biotech company, Revivicor (Blacksburg, VA), that had produced the first GTKO pigs a few weeks before Immerge. (Revivicor was the remnant of the Edinburgh [United Kingdom] company PPL Therapeutics that had successfully cloned the first large mammal - Dolly, the sheep [64].)

University of Pittsburgh

Jan Ijzermans' support of the xenotransplantation research program continued with the arrival of Pleunie Rood, and subsequently Dirk van der Windt and Eefje Dons from Rotterdam. Although Pleunie Rood's main contributions were related to pig islet transplantation in NHPs (see Bottino et al in this issue), she, together with other research fellows, carried out valuable *in vitro* studies determining the extent of anti-pig antibody binding to GTKO pig cells [65, 66]. Antibody binding was greatly reduced when Gal was not expressed on the pig cells.

Rood, and later Dons, carried out some challenging studies in infant baboons and demonstrated that treatment with an anti-CD154mAb inhibited the production of natural (preformed) antibodies [66, 67]. As natural antibodies (including both anti-ABO and anti-pig) had been considered to be T cell-independent, their data questioned this conclusion. If treatment was initiated soon after birth of the baboon (before anti-nonGal antibodies had begun to be produced), this resulted in baboons that developed *no* serum anti-GTKO pig antibodies until several weeks after cessation of anti-CD154mAb therapy [67]. This opened the possibility of carrying out pig organ transplants in human infants in the absence of anti-pig antibodies. This could include, for example, babies with life-threatening congenital heart disease for which no other therapy offered much chance of success (see below).

Working with Rita Bottino (Italy), Dirk van der Windt also concentrated much of his attention on islet xenotransplantation (see Bottino et al in this issue), but he also investigated alemtuzumab as an immunosuppressive agent in NHPs [68].

Later, GTKO pigs that expressed human complement-and/or coagulation-regulatory proteins became available from Revivicor. In collaboration with the late Agnes Azimzadeh (France), organs from these pigs were demonstrated to protect them further from the human (or NHP) immune response [69]. By this time, however, because the anti-CD154mAbs had been demonstrated to be thrombogenic [70], immunosuppression was achieved with an anti-CD40mAb, first introduced into xenotransplantation by Mohiuddin et al [71], but which proved not as effective as an anti-CD154mAb.

Burcin Ekser (born in Turkey and trained in Italy), a very productive Pittsburgh research fellow, working with liver transplant surgeon Bruno Gridelli (Italy, who had been active in xenotransplant research for some time), carried out some important studies of GTKO pig liver transplantation in NHPs [72, 73]. They demonstrated very clearly that within minutes a profound thrombocytopenia developed in the recipient which did not recover and ultimately resulted in internal bleeding, requiring euthanasia [73]. However, there was evidence of quite good function of the liver [72], suggesting that there was a potential for a pig liver to support a patient at least as a bridge until a liver from a deceased human donor became available.

University of Alabama at Birmingham

Enticed by the fact that Revivicor (by now owned by United Therapeutics) was building a 'clean' (designated pathogen-free) pig facility in Birmingham, Alabama, that would enable clinical pig organ xenotransplantation to proceed, members of the (by now "nomadic") research team relocated to Birmingham, where

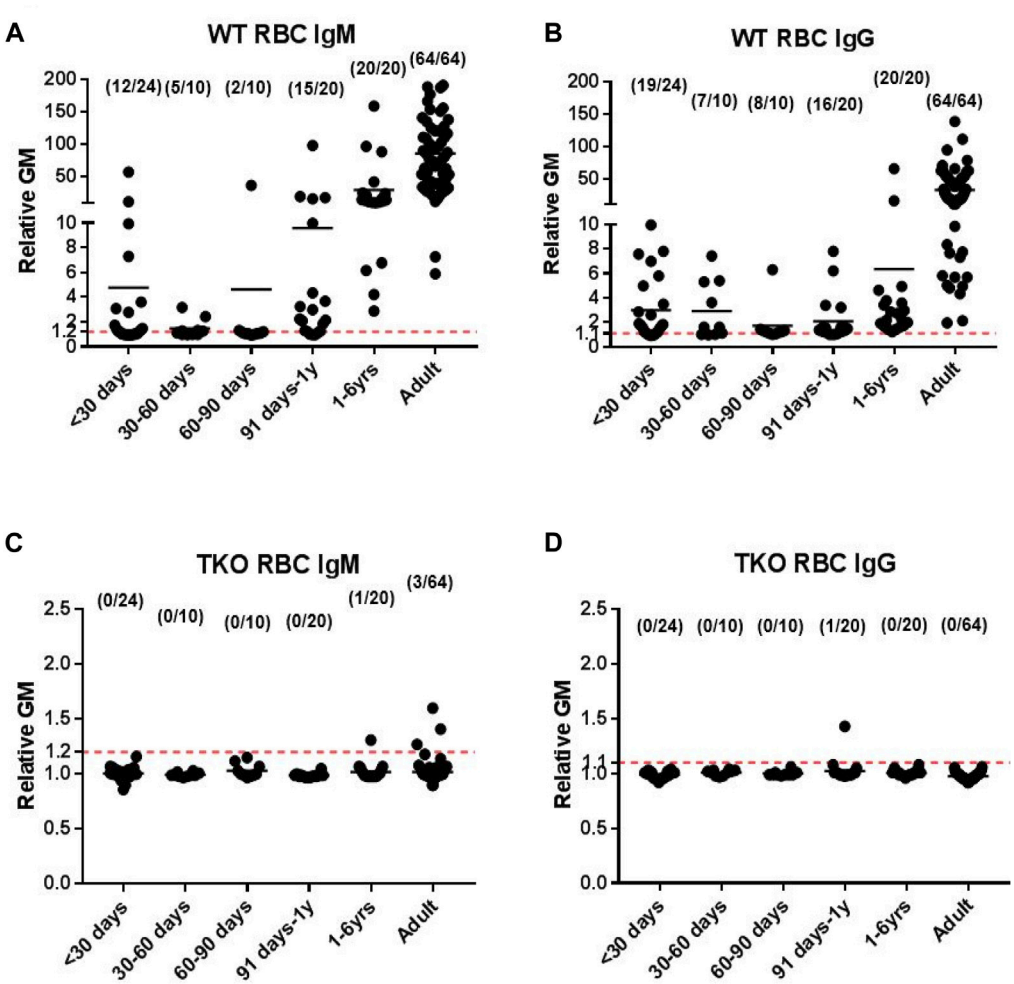


FIGURE 1 | Human serum IgM and IgG antibody binding to wild-type (WT) pig red blood cells (RBCs) (**A, B**, respectively) and triple-knockout (TKO) pig RBCs **(C, D)** at different ages. (Note the difference in the y-axis between binding to WT and TKO RBCs.) (Reproduced with permission from Li Q et al. Ann Thorac Surg. 2020; 109:1,268–1,273).

Joe Tector was also establishing a research program. Triple-knockout (TKO) pigs became available from Revivicor (**Figure 1**) but, despite also expressing six human transgenes and therapy with an anti-CD40mAb, the results of pig organ transplants remained mixed, in part due to the fact that all Old World NHPs have natural antibodies against these cells [74, 75].

Nevertheless, based on previous studies by this group (**Figure 1**) [66, 67, 76, 77], encouraging results of TKO pig orthotopic heart transplantation were obtained in collaboration with Birmingham pediatric cardiac surgeon David Cleveland, with survival of one baboon for almost 8 months [78, 79].

Sadly, when Devin Eckhoff, who had been active in establishing the xenotransplantation research program at UAB, moved to Boston, problems arose, and the team disbanded. Cooper moved back to the MGH where he continues an active research program today. An immunosuppressive regimen based on an anti-CD154mAb combined with rapamycin appears to prevent rejection in the TKO pig-to-baboon model [80].

Throughout these past 35 years intermittent efforts were also made to investigate the possibility of pig red blood cells being used for clinical transfusion. Under the mentorship of Frank Dor and other research fellows, visiting European students, such as Foad Rouhani (United Kingdom), played a significant role in these studies [81].

AGNES AZIMZADEH AND LARS BURDORF – PIG LUNG TRANSPLANTATION

As members of Robin Pierson's group, initially at Vanderbilt University, then at the University of Maryland, and subsequently at MGH, two Europeans, Agnes Azimzadeh (France) and Lars Burdorf (Germany), contributed very significantly to intensive studies of pig-to-baboon lung transplantation (as well as to many other studies). This model has been found to be extremely challenging and progress has been slow, with graft survival

still frequently measured in days rather than weeks [82]. Nevertheless, this group's dedicated efforts have made inroads in identifying the extreme immunological problems that prevent progress. Agnes Azimzadeh, in particular, also contributed to numerous other studies in xenotransplantation [83].

Several other Europeans (most commonly from Germany or France) were members of this group over the years, for example, Carsten Schroeder (Germany), with the most recent being Franzi Pollok (Germany), Ryan Chaban (Germany) [84], and Sara De Taeye (Belgium).

CHRISTOPHER MCGREGOR (UNITED KINGDOM) – PIG HEART TRANSPLANTATION

Chris McGregor, a British cardiothoracic surgeon and researcher, has for many years divided his time between the USA and United Kingdom. His initial research was largely directed towards pig heart xenotransplantation in NHPs and he collaborated with the U.S. biotech company, Nextran, whose research was largely directed by John Logan, also of Scottish origin. When at the Mayo Clinic, McGregor tested induction therapy with ATG and rituximab and maintenance therapy that included rapamycin in a tacrolimus-based immunosuppressive regimen; mean heart graft survival was extended longer than reported previously [85, 86]. Importantly, his close and long-term colleague, Guerard Byrne, identified the glycan Sda as the third carbohydrate xenoantigen against which humans have natural antibodies [87]. Its identification enabled triple-knockout pigs to be produced (**Table 2**), initially by Estrada et al. [74], moving xenotransplantation much closer to clinical experiments.

In recent years, McGregor and Byrne have concentrated their attention on testing gene-edited pig cardiac valve bioprostheses and established a pig-to-sheep model in Europe to do this [88]. These bioprostheses, at present based on GTKO pigs, should delay the deterioration seen in current pig cardiac valve bioprostheses after implantation in young patients.

THE MUNICH GROUP

A further group that has made major contributions to organ xenotransplantation is that from Ludwig Maximilians University in Munich, Germany. The early work of Claus Hammer (1940–2015), who held degrees in both medicine and veterinary medicine, related to the anatomical, physiological, and biochemical aspects of xenotransplantation, on which topic he was the world authority [89]. He also carried out interesting studies of concordant xenotransplantation, e.g., between fox and dog [90], and proved himself to be one of the most productive researchers in the 1980s and 1990s.

A new research endeavour at the university was established formally in 1998 by cardiothoracic surgeon, Bruno Reichart, to develop successful pig-to-baboon heart transplantation. It took several years for success to be achieved [91]. Difficulty in producing GTKO pigs was overcome by collaboration with Revivicor, but in recent years the Munich group has been a leader in the field. Its 'breakthrough' moment came in 2018 when it overcame the early pig cardiac graft failure that had prevented progress for several years [92], initially described and investigated by Chris McGregor's group [93]. This was suspected not to be associated with rejection; the data suggested it was due to a sensitivity of the pig heart to ischemia-reperfusion injury.

By using the Steen system of myocardial protection (a Swedish advance) [94], the problem was resolved and prolonged function (3–6 months) after pig orthotopic heart transplantation in baboons was consistently achieved for the first time [92]. (It appears, however, that, when the pig heart is small and the ischemic period is short, adequate myocardial protection can be achieved by static cold storage after cardioplegia with del Nido solution, which is much simpler and less expensive [79].)

Much of the success of the Munich group has been due to the work of Eckhard Wolf, an expert on gene-editing in pigs, who has also developed disease models for conditions such as diabetes mellitus and rare monogenic diseases [95, 96]. His work in tailored medical models in mice and pigs has led to innovative models to not only address the organ shortage but also improve treatment options for various diseases.

One innovation from this group was to demonstrate that, when the pig organ grew quickly after transplantation, thus leading to some compression within the NHP recipient, jeopardizing its function, deletion of the gene for growth hormone receptors reduced growth [97]. This problem, of course, has not been seen when the organs are taken from a miniature breed of pig.

HANNOVER MEDICAL SCHOOL AND FRIEDRICH-LOEFFLER INSTITUTE

A second group in Germany has been very active in the production of gene-edited pigs for xenotransplantation (and for several other reasons, e.g., as models of diseases), but less active in collaborating with surgical groups to transplant organs from these pigs in NHPs. This group, established in the 1990s and headed by veterinary scientist Heiner Niemann, and including well-known colleagues such as Bjorn Petersen and Reinhard Schwinzer, has contributed many innovations to the field over many years [98–102]. It has had a special interest in the introduction of 'anti-inflammatory' transgenes [103, 104].

PARALLEL STUDIES IN XENOTRANSPLANT IMMUNOLOGY

In parallel with these time-consuming and expensive studies in the pig-to-NHP model, several European laboratories were investigating aspects of xenotransplantation immunology by *in vitro* assays. Space does not allow a detailed discussion of their wide-ranging studies, but they included groups in the United Kingdom (London, led by Anthony Dorling and Robert Lechler) [105, 106], France (Nantes, Jean-Paul

Soulillou) [107], Switzerland (Jorg Seebach, Geneva/Zurich, and Robert Rieben, Bern) (see this issue). The Nantes group led by Gilles Blancho, also carried out studies in the pig-to-NHP model [108, 109]. Others include Eelco Bouwman (Netherlands), Beverly Hunt (United Kingdom), Luca Inverardi (Italy), Ignazio Marino (Italy), Ruggero Pardi (Italy), Leendert Paul (1946–2004, Netherlands and Canada), Raymond Reding (Belgium), Marlene Rose (United Kingdom), and Karen Ulrichs (Germany), among others.

THE POTENTIAL RISK OF XENOZOONOSIS

The potential risk of xenozoonosis has received much attention by experts in the field, and European infectious disease experts have played important roles. Joachim Denner (Germany) is one of the world experts on PERVs and has developed highly sensitive methods to detect potential xenozoonotic porcine viruses (see Denner J in this issue). His impact on virology and biomedical research has advanced knowledge on the safety of xenotransplantation. Others include Linda Scobie (United Kingdom) and Nicolas Mueller (Switzerland) (see Scobie & Mueller in this issue), Ralf Tonjes (Germany) (see Tonjes in this issue), and the late Henk Schuurman (Netherlands). Henk Schuurman, who spent much of his career commuting between Europe and the USA, put his knowledge into practice by overseeing the construction and functioning of the Spring Point project in Wisconsin, a facility built to breed and house pigs under designated pathogen-free conditions that would be acceptable to the US FDA [15].

In addition, Ralf and Henk played leading roles in advising participants and prospective participants on the regulatory aspects of xenotransplantation. Henk was perhaps unique in having an in-depth knowledge of regulation on both sides of the Atlantic. The regulatory landscape poses stringent requirements to mitigate the potential risks associated with the clinical use of xenografts, most notably the risk of xeno-zoonotic pathogen transmission [110]. Ralf and Henk have aided in navigating these regulations to create guidelines for clinicians and pave the way towards the development of safe and realistic xenotransplantation therapies [111, 112].

COMMENT

We thus conclude that over the past 50 years European surgeons and scientists have contributed significantly to progress in xenotransplantation research. Space has not allowed us to draw attention to all of the Europeans who have worked in this field, and we apologize for our omissions. We also apologize to the many non-Europeans who have contributed to the research reviewed in this article but who have not been identified.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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CONFLICT OF INTEREST

DC is a consultant to eGenesis Bio, Cambridge, MA, USA, but the opinions expressed in this article are those of the authors and do not necessarily represent those of eGenesis.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GENERATIVE AI STATEMENT

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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REVIEW
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Contributions of Europeans to Xenotransplantation Research: 2. Pig Islet and Cell Xenotransplantation

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Pig islet xenotransplantation in nonhuman primates (NHPs) has made considerable progress during the past 30 years, and European scientists in both Europe and the USA have contributed to this progress. At times, there have been, or are, active research programs in Sweden, Germany, Belgium, and the USA. The first clinical experiments of wild-type (i.e., genetically-unmodified) pig islet xenotransplantation were carried out by Groth and his colleagues in Stockholm in 1994, but without significant success. Hering's group in Minneapolis was the first to report prolonged survival of wild-type pig islets in NHPs in 2006, and the first report of insulin-independence for >12 months was by a "European" research team at the University of Pittsburgh in 2009. Recent progress has been slow, in part through a lack of funding, but recent advances in pig organ xenotransplantation suggest that pig islet xenotransplantation is poised for clinical experiments in the near future. In addition, there have been encouraging experimental studies of pig neural cell injections into the brains of monkeys with a pharmacologically-induced Parkinson's disease.

Keywords: Europeans, genetic engineering, pancreatic islets, pig, USA, xenotransplantation

INTRODUCTION

The contributions of Europeans to our understanding of pancreatic islets go back to their identification in 1869 by a German medical student, Paul Langerhans (1847–1888), although he did not know their function. The first attempt to transplant pancreatic tissue was in 1916 by the British surgeon, Frederick Pybus (1893–1975). We also need to consider the very controversial work in the 1920s and 1930s of Serge Voronoff (1866–1951), a Russian émigré in Paris who, although his work on testicular transplants was misguided, at least introduced the concept of transplanting xenogeneic cells that would provide replacement of native cells that had lost their function, e.g., insulin-producing islets.

The transplantation of porcine pancreatic islets for Type 1 diabetic patients has the potential to replace the current "gold standard" treatment of pancreatic allotransplantation. Many European researchers have contributed to advancing this area of xenotransplantation towards clinical reality,

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Abbreviations: GSMS, gastric submucosal space; GTKO, α1,3-galactosyltransferase gene-knockout; IBMIR, instant blood-mediated inflammatory reaction; mAb, monoclonal antibody; NHP, nonhuman primate.

with the potential to impact millions of patients living with diabetes globally – particularly so as the number of patients drastically out-numbers pancreas donors for allotransplantation, and frequently islets from two or more human donors are required for one recipient. Approaches to balance the impact of diabetes and the risks of immunosuppression have varied between (i) focusing on combined islet-kidney transplants (where immunosuppressive therapy is administered for protection of both the donor kidney and the islets), and (ii) encapsulation (or other physical isolation) of the islets to protect them from the recipient immune system without the need for immunosuppressive therapy.

RESEARCH IN SWEDEN

We must commemorate the late Carl-Gustav Groth, who, among his many contributions to kidney, pancreas, liver, and bone marrow allotransplantation in Sweden and globally [1], was the first to conduct clinical studies of porcine islet xenotransplantation. In 1994, Groth and his team at the Karolinska Institute undertook the xenotransplantation of wild-type fetal porcine islet-like cell clusters into insulin-dependent Type 1 diabetic kidney-transplant recipients [2]. The porcine cells were transplanted via the intraportal route and showed evidence of survival of some of them in the patients for several months. Urinary C-peptide, a marker of insulin production, was detected in some of the recipients. Groth's team used the renal subcapsular route of islet delivery in two recipients. (The renal subcapsular space has subsequently been explored for transplantation of combined "islet-kidneys.") Although none of the patients in Groth's original clinical study achieved normoglycemia (or even a reduced insulin requirement), his contribution was important in encouraging others to advance islet xenotransplantation towards the clinic.

The Swedish study served as a catalyst for multiple clinical trials of islet xenotransplantation conducted outside the EU, as detailed by Piemonti et al. (2024) [3]. In 2000 in New Zealand Elliot and his team transplanted non-genetically modified (wild-type) encapsulated neonatal pig islets into two patients. The study showed improved glucose metabolism while addressing concerns about porcine endogenous retroviruses (PERVs). In 2005 Valdes led a study in Mexico involving transplantation of wild-type neonatal pig islets combined with Sertoli cells in a collagen-covered device and no immunosuppression into 12 patients. Improved glucose metabolic control was reported. By 2011, Matsumoto and colleagues in New Zealand reported on the transplantation of encapsulated wild-type neonatal pig islets intraperitoneally into 14 patients in the absence of immunosuppression. The study demonstrated reduced hypoglycemic unawareness. During the same year, Wang's team in China transplanted neonatal pig islets from piglets negative for PERV-C into 21 patients via the intrahepatic route under immunosuppression. The results included reduced insulin requirements and improved HbA1c levels. In 2016, Matsumoto conducted another study in Argentina with eight patients. Neonatal pig islets (wild-type) were encapsulated and transplanted intraperitoneally without immunosuppression. This study demonstrated improved HbA1c and reduced hypoglycemic unawareness.

In 2018, Wang in China designed a clinical trial in accordance with Changsha regulations. Twenty patients received neonatal pig islets via the intraportal route, along with autologous T-regulatory cells and immunosuppression. The trial was successfully completed, highlighting advancements in immune regulation strategies to improve outcomes (unpublished).

Other Swedish researchers made major contributions to our knowledge of this field. Olle Korsgren's work at Uppsala University over many years on porcine islet biology and transplantation has been pivotal in advancing the field of islet xenotransplantation, and he played a key role in the design and implementation of Groth's pioneering study.

In subsequent studies with William Bennet, Korsgren identified a critical incompatibility between human blood and isolated islets, known as the instant blood-mediated inflammatory reaction (IBMIR), which significantly impacts the success of intraportal islet transplantation. They observed that both human and pig islets are damaged upon contact with human blood *in vitro* or when transplanted into the liver via the portal vein in nonhuman primates (NHPs). By dissecting the mechanisms behind the IBMIR, the Swedish team demonstrated that complement inhibitors and anticoagulants can reduce the damage to the islets [4, 5]. These findings contributed to a better understanding and modulation of IBMIR in the clinical setting of islet allotransplantation, helping improve the outcomes of both allo- and xeno-transplantation [6].

RESEARCH IN GERMANY

Significant contributions to the field of porcine islet xenotransplantation were made by members of the Justus-Liebig-Universität in Giessen, particularly in the areas of porcine islet isolation, preservation, and transplantation. The Giessen team, led by Reinhard Bretzel, developed methods to enhance the survival and function of porcine islets during cold storage and after transplantation into recipient animals [7]. Key advances by this team also improved the enzymatic digestion of the pancreas and the purification of porcine islets, along with refining the culture conditions that support the long-term functionality of the transplanted islets [8].

Moreover, the Giessen team shed light on the critical islet mass required to achieve successful metabolic function after transplantation in pig models [9]. These advances were crucial for increasing the number of islets available for experimental and preclinical studies, helping to make xenotransplantation more feasible.

Influenced by their work in Giessen, Bernhard Hering and Daniel Brandhorst moved from Germany to the USA and the UK, respectively, and further explored porcine islet isolation and transplantation and became prominent figures in the field of islet allo- and xeno-transplantation [10–13].

In Dresden, Germany, the group led by Barbara Ludwig has worked to optimize islet encapsulation technology. Their research

uses a variety of animal models, including the isolation of mouse, rat, pig, and human islets. Using a novel immuno-protective membrane, the group demonstrated restoration of normoglycemia, without the need for immunosuppression, in a model of rat islet xenotransplantation into diabetic minipigs [14]. Ludwig's team then showed success in NHP diabetic model, using porcine islet xenografts within a bioartificial pancreas device to create a suitable microenvironment for cell survival [15, 16]. Their BetaO2 device consists of islet compartments and a replenishable oxygen reservoir: a method which allowed better oxygenation and function of the islets, without immunological communication or pathogen transfer between the recipient and the donor graft. Ludwig and her researchers have taken steps towards safe clinical trials for patients with brittle diabetes to receive islet xenografts and continue to improve techniques in islet isolation and transplantation.

Other German groups have also contributed to investigations of porcine islet xenotransplantation by genetic engineering to reduce immune cell activation [17, 18].

RESEARCH IN BELGIUM

Under the leadership of Daniel Pipeleers at the Vrije Universiteit in Brussels, Marika Bogdani and colleagues investigated the growth and functional maturation of beta cells derived from endocrine implants of the prenatal porcine pancreas. Their research demonstrated that purified endocrine cells from the prenatal porcine pancreas could be implanted into hosts, successfully developing into mature, functional beta cells. This study highlights the critical importance of purifying endocrine cells from the prenatal porcine pancreas for successful transplantation [19].

At the Catholic University of Louvain, the work by Pierre Gianello and his team has significantly advanced the field of porcine islet xenotransplantation, particularly in improving islet isolation, addressing immune rejection, and exploring novel strategies like encapsulation. Denis Dufrane and Gianello demonstrated the survival of macro-encapsulated pig islets in NHPs without immunosuppressive therapy [20, 21]. Their patented alginate-coated collagen matrix device prevented entry of anti-pig antibodies, and allowed the islets to survive for up to 6 months. They refined critical factors, such as donor selection, isolation techniques, enzymatic digestion, and culture conditions, all of which enhance islet yield, viability, and functionality, making them more suitable for potential therapeutic use [20–22]. One of their innovative approaches relating to islet encapsulation has been successfully tested in preclinical studies involving NHPs, where they demonstrated a long duration of islet function, and aimed to optimize transplantation protocols for future clinical applications [23].

As with other areas of xenotransplantation, gene-modification strategies have also extended the limits of islet xenografts. Many European groups have delved into the development of the perfect gene-edited pig to yield the most suitable porcine islets. Pierre Gianello and Nizar Mourad published the first report of tailoring genetic modifications to increase insulin secretion from porcine islets [24, 25]. By modifying glucagon-like peptide-1 and type 3 muscarinic receptors in the pig, they were able to amplify glucose-stimulated insulin secretion [24]. With the important collaboration of Cesare Galli (Italy) (see below), they have now produced genetically-engineered pigs that produce significantly more insulin than any other pigs ([26], and see Mourad in this issue).

In a broader approach, Mourad and Gianello have also assessed the secretion of other pancreatic hormones, such as glucagon, noting both physiological similarities and challenges in matching human islet performance [26].

CESARE GALLI (ITALY) – GENE-EDITED PIGS

Cesare Galli is a prominent Italian researcher known for his groundbreaking work with gene-edited animals. He has over three decades of experience in assisted reproduction and biotechnology. He was actively involved in the development of nuclear transfer technology which led to significant milestones, such as cloning the progeny-tested bull Galileo in 1999, the first cloned horse, Prometea, in 2003, and the first cloned champion horse, Pieraz, in 2005 [27–29]. His team also successfully cloned piglets in 2006 and produced genetically-engineered pigs in 2008 by somatic cell nuclear transfer (SCNT) [30]. In 2009, he founded Avantea, which is internationally renowned in animal cloning. Galli's use of CRISPR-Cas9 technology has enabled the creation of pigs lacking the Gal and Neu5Gc xenoantigens but transgenically expressing human "protective" proteins, including CD46, CD55, and CD59, crucial for reducing hyperacute rejection in xenotransplantation [31]. In 2014, Galli co-founded Xenothera SAS to further explore the biomedical applications of gene-edited pigs.

CONTRIBUTIONS FROM EUROPEANS IN THE UNITED STATES

Research at the University of Minnesota

At the invitation of the well-known pancreas transplant surgeon, David Sutherland, Hering moved to the University of Minnesota, where he established a clinical islet allotransplantation program and experimental islet allotransplantation and pig islet xenotransplantation programs. By administering immunosuppressive therapy based on CD40/CD154 co-stimulation blockade [initially introduced into xenotransplantation research by Leo Buhler (Switzerland) and his colleagues in 2000], Hering's group was among the first to report prolonged survival of diabetic NHPs after wild-type pig islet transplantation [10, 32].

The University of Minnesota raised funding to build a new "clean" (i.e., a designated pathogen-free) pig facility to enable clinical trials of pig islet xenotransplantation to be initiated, and the late Hendrik-Jan Schuurman (Netherlands) was brought in to establish this facility (Spring Point), which he did successfully. However, to our knowledge, no clinical trial of porcine islet xenotransplantation has yet taken place.

In addition to his contributions to experimental islet xenotransplantation, Hering has actively participated in defining the role and objectives of the International Xenotransplantation Association in regard to islet xenotransplantation [33, 34].

Research at the University of Pittsburgh

In addition to Bernhard Hering and Hendrik-Jan Schuurman, other Europeans have worked in the USA either temporarily or permanently and have contributed significantly to progress in pig islet xenotransplantation. For several years, the Pittsburgh team included several research fellows from Jan Ijzerman's transplant center at Erasmus University in Rotterdam. The islet transplantation program, led by Rita Bottino (Italy) with strong support from Massimo Trucco (Italy), and David KC Cooper (Great Britain) made extensive efforts to advance our understanding of xenotransplantation and addressed key challenges in the field.

The Induction of Diabetes

The optimization of a safe and reliable model for inducing diabetes in cynomolgus monkeys, as achieved by Pleunie Rood (Netherlands), represented a significant advance in diabetes research [35]. This model utilizes clinical-grade streptozotocin, a drug known to target and destroy islet beta cells, leading to insulin deficiency, stable elevation of blood glucose levels, and the establishment of a diabetic state over time.

Interestingly, Nagaraju and Bottino observed that streptozotocin administration induced transient but substantial lymphopenia, accompanied by an increase in absolute monocyte count and serum monocyte chemoattractant protein-1 (MCP-1). These contrasting effects can influence immune responses following islet transplantation [36].

A deeper understanding of the systemic effects of streptozotocin in the early period after administration, combined with enhanced knowledge of glucose regulation in NHPs - particularly cynomolgus monkeys - has significantly reduced stress and improved safety, which is crucial for ethical considerations.

Glucose Regulation in Genetically Engineered Pigs and Metabolic Compatibility Across Species

Advances in managing glucose regulation before and after transplantation in NHPs receiving porcine islets were highlighted by Anna Casu (Italy) and her colleagues [37]. They investigated glucose metabolic responses in both pigs and NHPs and discussed their implications for clinical translation. Casu further examined differences in insulin secretion and glucose metabolism between α1,3-galactosyltransferase gene-knockout (GTKO) pigs and wild-type pigs [38]. GTKO pigs showed differences in insulin secretion compared to wild-type pigs. Although the genetic or molecular basis for these differences was not determined, the study concluded that these physiological variations are unlikely to significantly impact the outcome of GTKO porcine islet xenotransplantation.

In subsequent research, Martin Wijkstrom (Sweden), who had spent time working with Bernhard Hering in Minnesota, studied glucose metabolism in genetically-modified pigs expressing human transgenic proteins regulated by an insulin promoter [39]. These transgenic pigs exhibited improved blood glucose regulation compared to wild-type pigs, suggesting that human gene expression under an insulin promoter enhances the potential of these pigs as sources of islets for xenotransplantation.

Loss of Islets From the IBMIR

As described initially by Bennet and his colleagues (see above), substantial islet loss often occurs early after their intraportal transplantation due to the IBMIR. Various strategies have been explored to mitigate these effects, including the administration of biologics (e.g., cobra venom factor) and pharmaceuticals (e.g., dextran sulfate) that inhibit complement activation, modulate coagulation, and reduce inflammation. Additionally, transgenic expression of proteins in donor pig islets that provide some protection from human complement and coagulation activation has proven effective in reducing the effects of IBMIR [40, 41].

In an *in vitro* model of IBMIR, Dirk van der Windt (Netherlands) investigated the mechanisms underlying early islet graft damage and found that humoral immunity played a more important role in triggering and exacerbating IBMIR than previously recognized (**Figure 1**) [42]. In similar settings, using neonatal islet-like cell clusters from wild-type and GTKO pigs expressing human CD46 (GTKO/CD46) or CD46 and CD39 (GTKO/CD46/CD39), Nagaraju et al. demonstrated that neonatal islet-like cell clusters experienced humoral injury regardless of their genetic modifications [43].

Intraportal Adult Pig-To-NHP Islet Transplantation

Successful long-term survival of islet grafts was achieved by Dirk van der Windt and his colleagues through a combination of strategies, including optimized diabetes induction in cynomolgus monkeys, an anti-inflammatory protocol, an immunosuppressive regimen based on an anti-CD154 monoclonal antibody (mAb), and the use of pig islets expressing the human complement regulator CD46 [44]. This was the first report of pig islet transplantation resulting in insulin-independence and normoglycemia for a period of >12 months (**Figure 2**). This significant achievement was later replicated using GTKO pig donors that expressed multiple human transgenes targeting complement and coagulation activation and inflammation [46]. It demonstrated the beneficial effects of tissue targeted gene editing in modulating IBMIR and early islet loss, thus advancing the acceptance of genetically-engineered porcine tissues for transplantation. However, in neither study were the results consistently successful.

Conducting pre-clinical trials with long-term follow-up enabled the refinement of methodologies for monitoring diabetes in NHPs post-transplantation. Marco Marigliano (Italy) and his colleagues demonstrated that monitoring hemoglobin A1C is a reliable metric for assessing glucose control both before and after islet xenotransplantation [47].

Standardization of experimental conditions ensured the overall safety of the procedure. However, complications were reported. For example, Nathalie Campanile (Switzerland)

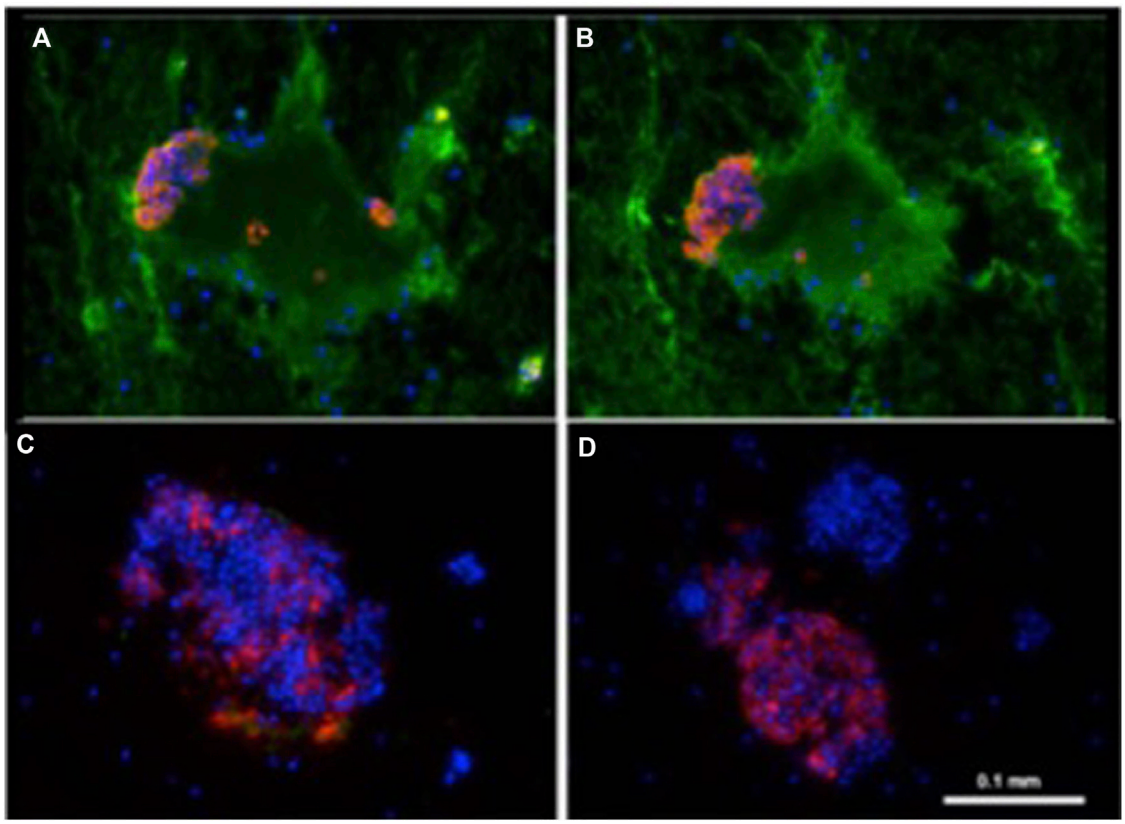


FIGURE 1 | Binding of human IgM and IgG antibody to pig islets (xenogeneic) **(A, B)** and to human islets (allogeneic) **(C, D)**. IgM [green, **(A, C)**], IgG [green, (B, D)], insulin (red), nucleus (DAPI/blue). Yellow indicates colocalization of insulin and IgM/IgG. Adapted with permission from "Binding of human IgM and IgG antibody to pig islets (xenogeneic) **(A–B)** and to human islets (allogeneic) **(E–F)**. Binding of pig IgM and IgG antibodies to pig islets (autologous) **(C–D)**. IgM (green, A, C, E), IgG (green, B, D, F), insulin (red), nucleus (DAPI/blue). Yellow indicates colocalization of insulin and IgM/IgG" by Dirk J. Van Der Windt, Marco Marigliano, Jing He, Tatyana V. Votyakova, Gabriel J. Echeverri, Burcin Ekser, David Ayares, Fadi G. Lakkis, David K. C. Cooper, Massimo Trucco and Rita Bottino, licensed under CC BY-NC.

reported that, 1 week after intraportal pig islet transplantation, one recipient developed acute gastric dilatation that proved fatal [48]. Acute gastric dilatation had been described before in NHPs and has subsequently proved problematic in baboons after pig organ transplantation. The report by the Pittsburgh group offered guidelines for preventing or managing acute gastric dilatation.

Rita Bottino and Angela Criscimanna (Italy) reported interesting findings related to the recovery of beta cell mass and function in NHPs that had been rendered diabetic with streptozotocin and subsequently received porcine islet transplants [49]. Although the return of native beta cell function following the induction of hyperglycemia and islet transplantation in large animal models is rare, it can be a significant confounding factor if not properly detected, potentially impacting the interpretation of islet graft performance. The recovery of native beta cell function underscored the need for mechanistic investigations to explore the regenerative properties of pancreatic cells and the potential role of transplantation. It also highlighted the importance of rigorous monitoring of islet graft performance and the selection of testing methods that can distinguish between endogenous and donor islet metabolic responses.

Exploration of Immunosuppressive Agents in NHPs

Alemtuzumab (Campath 1H) is a potent immunosuppressive antibody that targets CD52, a molecule expressed on lymphocytes in humans and some NHPs. However, in many NHPs CD52 is also expressed on erythrocytes which are hemolyzed after the administration of alemtuzumab. A small subset of cynomolgus moneys (from Indonesia) do not express CD52 on their erythrocytes and are, therefore, suitable for studies of alemtuzumab.

Although not specific to porcine islet xenotransplantation, Dirk van der Windt (working with Fadi Lakkis) investigated the effects of alemtuzumab on lymphocyte depletion and subsequent repopulation in Indonesian cynomolgus monkeys [50]. Alemtuzumab effectively depleted all circulating lymphocytes in the monkeys. This depletion was both rapid and profound – more profound than after the administration of anti-thymocyte globulin - impacting both T and B cells. Lymphocyte repopulation occurred gradually over time, the repopulation patterns varying among different lymphocyte subsets, with some recovering more quickly than others. The study provided valuable insights into the kinetics of lymphocyte depletion and repopulation.

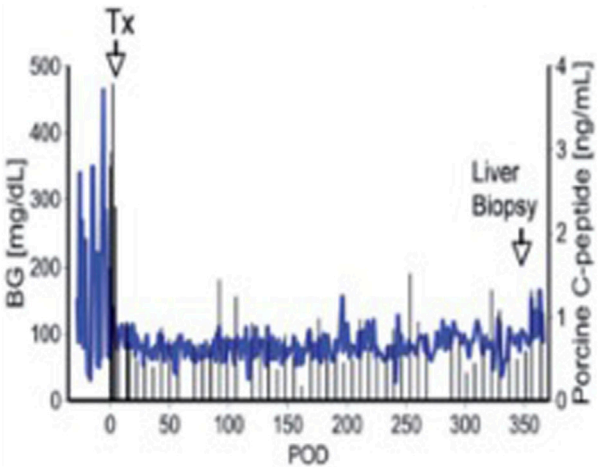


FIGURE 2 | Blood glucose and pig C-peptide levels in a streptozotocin-induced diabetic cynomolgus monkey before and after intraportal transplantation of islets from a pig expressing the human complement-regulatory protein, CD46. No exogenous insulin was administered after the transplant. The normoglycemic monkey was electively euthanized after 12 months. Day 0 = day of islet transplantation. Adapted with permission from "Blood glucose and pig C-peptide levels in a streptozotocin-induced diabetic cynomolgus monkey before and after intraportal transplantation of islets from a pig expressing the human complement-regulatory protein, CD46. No exogenous insulin was administered after the transplant. The normoglycemic monkey was electively euthanized after 12 months. Day 0 = day of islet transplantation" by Cooper D. K.C. and Bottino R., licensed under CC BY-NC-SA 4.0.

It was well-known that the anti-CD154mAbs available more than 10 years ago were thrombogenic in NHPs with organ allografts or xenografts [51–53]. However, after pig islet transplantation, the administration of an anti-CD154mAb was found to be both effective and safe [54]. While trials in humans initially identified thromboembolic events as a safety concern, these were not clinically or microscopically observed in a study of >20 cynomolgus monkeys receiving pig islet grafts. No evidence of thrombosis or thromboembolism was documented despite careful histopathological examination, including of the brain. It was hypothesized that the exposure of the recipient to the very small antigen load of islets (when compared with a whole organ) may have been insufficient to induce thrombosis.

Furthermore, no other adverse effects were reported. These findings supported the use of anti-CD154mAb as a promising strategy for enhancing the survival and function of pig islet transplants in NHPs, potentially warranting reconsideration for human use. However, the subsequent introduction of Fc-modified anti-CD154mAbs that have been designed to prevent any thrombogenic effect has ensured their safety in this respect.

The Gastric Submucosal Space (GSMS) as an Alternative Site for Islet Transplantation

As a preliminary for studies in the pig-to-NHP model, the GSMS was explored as a site for pig islet allotransplantation. The GSMS is an accessible location and has a rich vascular supply and may have potential advantages over the traditional intraportal site for islet transplantation. Gabriel Echeverri and colleagues developed a technique for transplanting islets into the GSMS using endoscopy [55]. This approach demonstrated potential for reversing diabetes in diabetic pigs, as evidenced by reduced blood glucose levels. Further studies by Fujita and Tanaka confirmed the feasibility of obtaining endoscopic biopsies from this site and also demonstrated that these biopsies allowed for detailed histological examination [56, 57]. However, in a limited clinical trial by James Shapiro (UK and Canada) and his colleagues, the GSMS was found to be less successful than hoped [58].

PIG NEURAL CELL XENOTRANSPLANTATION IN THE TREATMENT OF PARKINSON'S DISEASE

In an effort to investigate whether pig neural cell precursors could prove to be a possible treatment option for neurodegenerative disorders, Aron Badin, Cozzi, Hantraye, and their colleagues conducted very interesting studies in NHPs with a pharmacologically-induced Parkinson disease-like condition [59]. They transplanted into immunosuppressed Parkinsonian monkeys, neural precursors from either wild type or genetically-engineered pigs in which fetal mesencephalic neurons expressed CTLA4-Ig.

The results obtained in these studies were impressive. NHPs with xenografts showed an extraordinary recovery of locomotor function that persisted for as long as 31 months in one recipient of a CTLA4-Ig porcine embryonic graft. Furthermore, such a clinical recovery was associated with restoration of dopaminergic activity as demonstrated by positron emission tomography and histological examination. In addition, these studies demonstrated that, notwithstanding the brain is considered an immuno-privileged site, at least in this model systemic immunosuppression appeared indispensable to achieve long-term neural xenograft survival.

Unfortunately, these exciting and encouraging studies had to be discontinued when certain countries in Europe banned research involving NHPs.

XENOTRANSPLANTATION OF BONE MARROW CELLS AND HEPATOCYTES

Leo Bühler (Switzerland) has dedicated much of his career to advancing the clinical feasibility of xenotransplantation and overcoming its associated immunological challenges. His contributions span multiple aspects of the field, including hematopoietic cell, islet, and hepatocyte xenotransplantation, as well as developing frameworks for safe and ethical xenotransplantation clinical trials.

Bühler's early studies focused on hematopoietic cell transplantation in the pig-to-nonhuman primate (NHP) model [60]. It was when studying this challenging problem that he first demonstrated that co-stimulation blockade of the CD40/CD154 pathway provided better immunosuppression than conventional therapy. These investigations emphasized the importance of using appropriate anti-rejection, antithrombotic, and anti-inflammatory agents to address complications [61, 62].

TABLE 1 | Summary of key contributions to cell and islet xenotransplantation research by country and researcher/team.

Country	Researcher/Team	Contributions
Sweden	Carl-Gustav Groth	Conducted first clinical studies on porcine islet xenotransplantation; developed intraportal and renal subcapsular transplantation techniques [1, 2]
	Olle Korsgren, William Bennet	Identified IBMIR; studied porcine islet biology; developed complement inhibitors and anticoagulants to mitigate IBMIR damage [4, 6]
Germany	Reinhard Bretzel Daniel Brandhorst	Improved porcine islet isolation, enzymatic digestion, and culture; studied critical islet mass for transplantation success [7–12]
	Barbara Ludwig	Developed islet encapsulation technologies; demonstrated normoglycemia restoration using bioartificial pancreas devices [14–16]
Belgium	Daniel Pipeleers Marika Bogdani	Explored the functionality of porcine islets for xenotransplantation, insulin secretion dynamics and compatibility with human systems [19]
	Pierre Gianello Denis Dufrane Nizar Mourad	Improved islet isolation, encapsulation, and viability; developed alginate-coated collagen matrix for macro-encapsulation; enhanced genetic modifications for insulin secretion [20–26, 67]
Italy	Cesare Galli	Pioneered gene-edited pigs using CRISPR-Cas9; developed pigs expressing human complement regulators to reduce hyperacute rejection [27–31]
	Emanuele Cozzi	Explored neural precursor transplantation for Parkinson's treatment; demonstrated long-term recovery of locomotor function in NHP models [59]
USA (Europeans)	Bernhard Hering (Germany)	Advanced islet allotransplantation and xenotransplantation; developed clean pig facilities; studied anti-CD154 mAb immunosuppressive strategies [10–13, 32–34]
	David KC Cooper (Great Britain) Rita Bottino (Italy)	Investigated beta-cell recovery and IBMIR effects; developed diabetes models in NHPs; optimized transplantation techniques [35, 36, 46, 49]
	Dirk van der Windt (Netherlands)	Explored IBMIR mechanisms and mitigation strategies; achieved long-term survival of pig islet transplants in NHPs [41, 42, 44, 50]
	Marco Marigliano (Italy)	Standardized hemoglobin A1C monitoring for glucose control in preclinical transplantation studies [47]
	Anna Casu (Italy)	Investigated glucose metabolism differences between genetically modified and wild-type pigs and intraspecies compatibility [37, 38]
	Martin Wijkstrom (Sweden)	Researched glucose metabolism in genetically modified pigs expressing human transgenes regulated by insulin promoters [39]
	Nathalie Campanile (Switzerland)	Reported complications of intraportal porcine islet transplantation in NHP [48]
	Pleunie Rood (Netherlands)	Achieved safer induction of diabetes in NHP and contributed to reduce islet graft loss following intraportal porcine islet transplantation [35, 40]
Netherlands	Henk Shuurman	Contributed to advancement in ethical, regulatory, and preclinical aspects of xenotransplantation [10, 13]
Switzerland	Leo Buhler	Explored hematopoietic and hepatocyte xenotransplantation; developed immunological strategies for clinical trials [60–66]

His research subsequently extended to hepatocyte transplantation, where he explored innovative methods to treat acute liver failure through xenotransplantation [63, 64]. His team investigated the transplantation of encapsulated hepatocytes in both mice and baboons, revealing survival benefits without stimulating native liver regeneration. Further work included the development of strategies of co-encapsulation of hepatocytes with mesenchymal stromal cells (MSCs) to improve porcine hepatocyte viability and functionality [65].

Leo has also made major contributions to the field of xenotransplantation by (i) his long-term role as editor of the official journal of the International Xenotransplantation Association (IXA) and (ii) by maintaining a registry of the clinical transplants carried out in the world [66].

Table 1 summarizes the significant contributions detailed above.

COMMENT

As with our accompanying review of the contributions of Europeans to research into pig organ xenotransplantation, space has not allowed us to draw attention to all of the Europeans who have worked on islet or cell xenotransplantation, and we apologize for our omissions. We also apologize to the many non-Europeans who have contributed significantly to the research reviewed in this article but who have not been identified. The advances we have reviewed provide evidence for the contributions of European surgeons and scientists, in collaboration with their non-European colleagues, to the potentially important field of islet xenotransplantation.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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CONFLICT OF INTEREST

RB is an employee of Imagine Pharma Pittsburgh PA, United States, and DKCC is a consultant to eGenesis Bio, Cambridge, MA, United States, but the opinions expressed in this article are those of the authors and do not necessarily represent those of the companies.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GENERATIVE AI STATEMENT

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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REVIEW
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Enhanced Insulin Production From Porcine Islets: More Insulin, Less Islets

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Clinical pancreatic islet xenotransplantation will most probably rely on genetically modified pigs as donors. Several lines of transgenic pigs carrying one and more often, multiple modifications already exist. The vast majority of these modifications aim to mitigate the host immune response by suppressing major xeno-antigens, or expressing immunomodulatory molecules that act locally at the graft site. While these modifications are essential and have proven beneficial in preclinical trials, ensuring good intrinsic islet secretory function is equally important to achieve normoglycemia in recipients. Neonatal and even adult porcine islets are known for their low secretory response to physiological stimulation, a shortcoming that is often overcome by implanting extremely large numbers of such islets to compensate for insulin requirement incompatibilities between donor pigs and rodent, non-human primate or human recipients. Recent studies have revealed the existence of secretory amplifying pathways in porcine beta-cells previously identified in murine and human cells. Building upon these findings, a new line of transgenic pigs where these pathways are activated specifically in beta-cells has been created. Compared to their wild-type counterparts, islets from these transgenic pigs have proven to be better insulin secretors in their native pancreas environment, *in vitro* after isolation and most importantly *in vivo* after transplantation to diabetic mice.

Keywords: porcine islets, insulin secretion, transplantation, genetic modification, diabetes

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INTRODUCTION

Islet replacement therapies aiming to restore insulin independency in type I diabetic patients need to ensure that transplanted cells from human or animal origin produce enough insulin to sustain the host's physiological needs and establish efficient glucose homeostasis. This is also true even when the therapy's ambitions are limited to improving the patient's quality of life by diminishing exogenous insulin doses or alleviating the risk of severe hypoglycemia as seen in difficult-to-manage brittle diabetes. Surgical and immunological considerations such as the host immune response, implantation site, graft vascularization and its access to nutrients and oxygen as well as its capacity to deliver the secreted hormones all weight in the successful outcome of the procedure but intrinsic secretory properties of the grafted cells remain an undeniably determinant factor. From a qualitative point of view, porcine insulin is almost identical to its human counterpart differing only by one amino acid at position 30 in the B-chain (alanine in pigs vs. threonine in humans) [1]. Quantitatively, insulin secretion from porcine islets is significantly low compared to secretion from human islets in response to the same stimuli [2]. While everybody agrees on this specificity of porcine islets, different strategies have been proposed to overcome this limitation with most research groups resorting to increasing the number of implanted cells to compensate low secretion, promoting cell

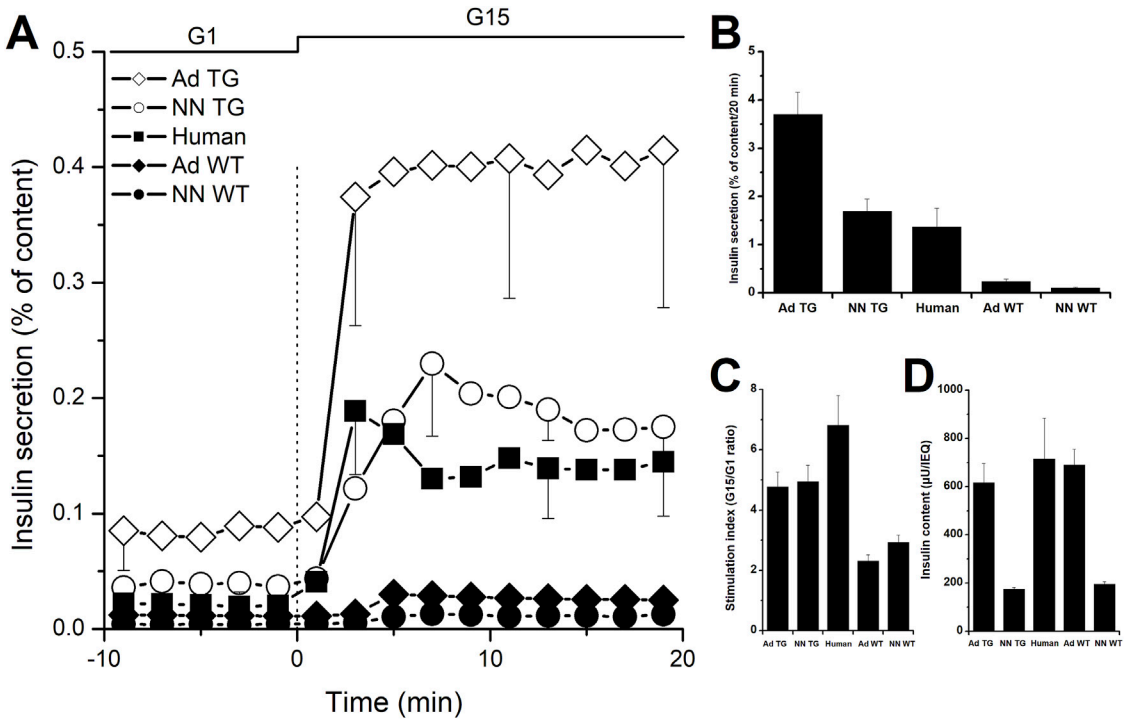


FIGURE 1 | *In vitro* insulin secretion from wild-type and InsGLP-1M3R isolated islets. Dynamic islet perifusion experiments were performed to evaluate islet secretory response. **(A)**: batches of 1000 IEQ islets from neonatal (NN) wild-type (WT, closed symbols) and InsGLP-1M3R (TG, open symbols) piglets or 200 IEQ islets from adults (Ad) were perifused with 1 mM glucose (G1) then stimulated with 15 mM glucose (G15) as shown on top of the figures. Similar experiments performed using human islets (closed squares) are shown for comparison. **(B)**: Area under the curve integration of insulin secretion during 20 min of G15 stimulation is represented. **(C)**: Stimulation indices (G15/G1 ratio) are shown. In all experiments, islets in perifusion chambers were retrieved at the end of the experiments and lysed to determine total insulin content shown in **(D)**. Results are expressed as percentages of total islet insulin content. Values are means ± SE from n = 10–50 experiments.

differentiation and maturation *in vitro* especially when using neonatal islets or using genetic modifications to improve the secretory capacity of porcine islets as we have recently suggested. In the present review, we briefly summarized our current knowledge of porcine islet physiology, their efficacy in experimental xenotransplantation models with particular emphasis on the development of genetically modified, functionally enhanced islets.

GLUCOSE HOMEOSTASIS AND INSULIN REQUIREMENTS IN PIGS, HUMANS AND NON-HUMAN PRIMATES

In the context of xenotransplantation, immune incompatibilities seem like the most obvious barrier hindering graft survival and its subsequent function. Beyond this, functional physiological compatibility between donor and recipient species is equally decisive. It is therefore worthy looking at differences and similarities of glucose regulation in species of interest. During an intravenous glucose tolerance test (IVGTT), the quick rise in glycaemia is paralleled by a rapid sevenfold increase of insulinemia in macaques compared to a much slower lingering rise in pigs [3]. Moreover, fasting glucose was found to be lower whereas insulin and c-peptide were significantly higher in non-human primates compared to pigs [4]. In humans, the situation is intermediate although somehow closer to that of pigs regarding glycaemia and c-peptide values [4]. Interestingly, in pigs, the impact of glucose changes on glucagon secretion seems greater and faster to occur than on insulin secretion [5]. These discrepancies suggest a relative physiological incompatibility between islets from porcine and human origin and even more so with non-human primates. Quantitative and/or qualitative adaptations of porcine islets seem then necessary to compensate for this shortcoming.

WHAT DO IN VITRO STUDIES TELL US?

Glucose-stimulated insulin secretion from isolated islets can be thoroughly studied *in vitro* using static incubation and/or dynamic perifusion assays therefore providing insight into the function of these islets that can be predictive of their capacity to alleviate or heal diabetes once transplanted. Ideally, grafted islets should secrete the same amount of insulin as the host's native islets when exposed to similar stimuli. However, we know that porcine islets secrete down to 10-times less insulin compared to human or non-human primates islets when

challenged with 15 mM glucose as evidenced by a lower stimulation index (the ratio of stimulated to unstimulated secretion; **Figure 1C**) and a much smaller area under the curve (AUC) for insulin secretion curves (**Figure 1B**) [6–10]. Unsurprisingly, the difference between porcine and human islets becomes even more striking when using islets from neonatal piglets as their immature cells contain less insulin and therefore secrete less of it as previously shown in our own [6] and other studies [9, 11–15]. Interestingly, such islets present the advantage of undergoing further maturation *in vitro* especially if cultured under optimized conditions, receiving growth and maturation factors from a specific culture medium and physical and structural support from encapsulation on a collagen matrix [16].

From a qualitative point of view, the secretory response of porcine islets to glucose stimulation is characterized by a relatively small first phase, an ascending second phase (**Figure 1A**) as well as a paradoxical off-response i.e., an unexpected short-lived increase of insulin secretion upon return to a resting glucose concentration [17]. Some have attributed this to the small number of alpha-cells in porcine islets (8% vs. 30% in porcine and human islets respectively) [18], the majority of which is further lost during isolation due to their peripheral localization and the lack of a peri-insular capsule [19]. However, this hypothesis has been challenged by more recent findings showing similar composition and distribution of alpha-cells in human and porcine islets [20]. Nonetheless, glucagon has been shown to amplify insulin secretion from porcine islets [21]. However, this cannot explain low insulin secretion from native porcine islets *in vivo* as previously mentioned.

A few studies attempted to investigate mechanisms that can potentially explain the weak secretory response of pig islets. However, no defects were found in glucose sensing, transport, phosphorylation and utilization which were all comparable to what is observed in rodent and human islets [22, 23]. On the other hand, we [2] and others [24] have observed that glucose-induced cytosolic calcium changes in porcine beta-cells are small and delayed compared to rodent [25] and human [26] islets and that typical glucose-induced calcium oscillations, a hallmark of stimulus-secretion coupling in pancreatic beta-cells, were not observed in individual porcine islets [2].

Glucose also modulates the amplitude of the insulin secretory response via a non-calcium-dependent mechanism known as metabolic amplification [27]. This mechanism first investigated in rodents and later demonstrated in human islets [28] has been shown to be equally operational in porcine islets as demonstrated by experiments using ATP-dependent potassium channels (KATP) modulators [7]. In most of the aforementioned experiments, exposure of porcine islets to adenylyl cyclase activators or other pharmacological substances inducing an increase of cAMP concentrations always resulted in a significantly increased secretory response to glucose stimulation, a principle that we recently utilized to produce modified functionally enhanced porcine islets as discussed later in this review.

PIG ISLET XENOTRANSPLANTATION

In the research setup, two main models have been used to evaluate efficacy of porcine islets in treating or alleviating chemically- or surgically-induced type-I diabetes: pig-to-non-human primate and pig-to-rodent. While both models have their pros and cons, they both represent a particularly challenging environment for porcine islets. As discussed earlier, non-human primates have higher insulin requirements than both pigs and humans [4] while rodent insulin differs substantially from its porcine counterpart [29] lessening efficacy of the latter in mice and rats. Therefore, one could expect porcine islets to underperform in both cases. This is particularly exemplified by the extremely large number of islets that are necessary to exert an effect in such experiments. Nonetheless, several studies have reported successful long-term reversal of diabetes following transplantation of free [30–37] or encapsulated [38–40] porcine islets in preclinical non-human primate models.

As indicated in **Table 1**, transplanted islet dose ranged from 10,000 to 100,000 IEQ/kg recipient body weight when using adult pigs as islet donors and 9,000 to 115,000 when using islets from neonatal piglets. Free islets are usually injected in the portal vein, thus mimicking clinical practice in human islet allotransplantation but exposing islets to IBMIR known to cause substantial early islet mass loss and impaired secretory function due to mitochondrial damage in surviving islets [41]. On the other hand, encapsulated islets are transplanted subcutaneously or in the peritoneal space where the main limitation is oxygenation [39] and access to nutrients during the critical period between implantation and graft vascularization.

Immunosuppression whether administered systemically to the recipient and/or created locally by means of genetic modification to the donor cells can help alleviate IBMIR and other immune rejection mechanisms [42] therefore improving transplantation outcome and even decreasing the number of islets required for efficient diabetes reversal [37]. In the case of encapsulated islets, oxygen supplementation either directly to the encapsulation device [38] or indirectly by pre-vascularizing the graft site or including angiogenic factors in encapsulation gels can help minimize islet loss due to hypoxia.

The pig-to-rodent model is easier to implement due to obvious cost-related as well as regulatory and ethical considerations and has been extensively used to refine experimental procedures before moving on to large animal models. Another advantage of mice models is the availability of immunodeficient mice strains permitting xenotransplantation studies without possible interference from immunosuppressant treatments. As such, these studies have focused more on finding the optimal implantation site or engineering it [43, 44]. The kidney subcapsular space is often used as a golden standard implantation site in these experiments using free islets [43, 45, 46] whereas encapsulated islets are typically implanted in the peritoneal [47, 48] or subcutaneous [44] space.

As shown in **Table 1**, mice received doses of up to 400,000 IEQ/kg [47], the minimal reported dose being 20,000 IEQ/kg [44]. In this context, an interesting approach was to adapt the transplanted islet dose to the daily insulin requirements of diabetic rat recipients. Agarose-encapsulated

TABLE 1 | Experimental pig-to-NHP and pig-to-rodent islet xenotransplantation.

Recipient	Donor age	Site	Islet dose (IEQ/kg)	1st author	Year	Ref
Cynomolgus	Adult	SC	20,000	Ludwig	2017	37
Cynomolgus	Adult	P	48,000–91 000	Safley	2018	38
Rhesus	Adult	IP	77,000–100 000	Shin	2018	30
Rhesus	Adult	IP	87,000–100 000	Min	2018	31
Rhesus	NN	IP	39,000–115 000	Gao	2021	32
Rhesus	Adult	IP	55,000–100 000	Kim	2021	33
Cynomolgus	Adult	IP	20,130	Graham	2022	35
Baboons	NN	IP	9,000–56 000	Hawthorne	2022	36
Cynomolgus	Adult	P	9,800–22 900	Holdcraft	2022	39
Mice	NN	P	80,000	Kobayashi	2006	47
Mice	NN	KC/IM	100,000–120 000	Wolf-van Buerck	2015	44
Mice	Adult	SC	20,000	Yu	2020	43
Mice	Adult	P	400,000	Ajima	2023	46
Mice	NN	KC/SC	32,000–160 000	Citro	2023	42
Mice	NN	KC	100,000	Mourad	2024	45

Non-human primates (NHP) and mice received pancreatic islets from adult or neonatal (NN) pigs. Transplanted islet dose is indicated as IEQ/kg recipient body weight. For mice experiments, IEQ/kg was calculated assuming a body weight of 25 g/mouse. Islets were transplanted in the portal vein (IP), subcutaneously (SC), in the peritoneal space (P), under the kidney capsule (KC) or in an intramuscular (IM) site.

neonatal porcine islets were incubated *in vitro* to determine their daily insulin output and the number of macrocapsules to be implanted was adjusted to equal the amount of insulin units necessary to maintain normoglycemia [49].

GENETIC MODIFICATIONS TO IMPROVE PORCINE ISLET INSULIN SECRETION

As mentioned earlier, most *in vitro* studies successfully used cAMP-increasing molecules to amplify glucose-induced insulin secretion from porcine islets [7] suggesting that insulin synthesis and content are not limiting factors. Indeed, intra-islet insulin content is similar in adult porcine and human islets but expectedly 3-times lower in neonatal piglet islets as shown in **Figure 1D**.

From numerous studies using murine islets and a growing number of studies using human islets, it has been established that hormonal and neural amplifying pathways exist in pancreatic beta-cells. Agents that bind GLP-1 receptors or directly activate adenylyl cyclase induce a rise in cAMP leading to activation of protein kinase-A (PKA) and Epac2, two protein kinases known to amplify glucose-stimulated insulin secretion mainly by increasing the number of readily releasable insulin granules. The parasympathic cholinergic neural amplifying pathway involves binding of acetylcholine to type-III muscarinic receptors (M3R), activation of membrane phospholipase leading to accumulation of diacylglycerol in the cytosol which activates protein kinase-C (PKC), another protein kinase involved in priming insulin granules to render them readily releasable. Using chemical activators of these pathways, we demonstrated that they exert an amplifying effect on porcine islet insulin secretion and even reported an unexpected synergetic effect when both pathways are activated in neonatal and adult porcine islets by means of pharmacological agents or adenoviral expression of a mutated GLP-1 and a constitutively activated form of M3R(6). In this model, substituting alanine at position 8 by a serine in the GLP-1 sequence prolongs the peptide half-life by rendering it resistant to dipeptidyl peptidase whereas a single-point mutation at position 490 in the M3R sequence renders the receptor constitutively activated [6]. Putting these two sequences under a porcine insulin promoter, we created an expression cassette (InsGLP-1M3R) that was then used to generate a line of transgenic pigs with beta-cell targeted expression [46].

As shown in **Figure 1A**, neonatal and adult islets isolated from these pigs secrete 16- to 15-times more insulin respectively compared to their wild-type counterparts. Compared to human islets, fold-increases are 1.2 and 2.7 for neonatal and adult InsGLP-1M3R islets respectively as illustrated in **Figure 1B**. In terms of stimulation index calculated as the ratio of secretion at high glucose to that at low glucose, InsGLP-1M3R islets were more responsive to glucose stimulation 4.9 vs. 2.9 for neonatal islets and 4.8 vs. 2.3 for adult islets. In comparison, we calculated an index of 6.8 for human islets (**Figure 1C**).

Recently, we reported using InsGLP-1M3R islets to treat streptozotocin-induced diabetes in immunodeficient mice [46]. These experiments show that 100% of InsGLP-1M3R islet recipients became normoglycemic following islet transplantation under the kidney capsule. In comparison, only 22% of wild-type islet recipients were successfully treated. Despite implanting the same amount of both islet types (2500 IEQ), we observed significantly higher porcine c-peptide values in the InsGLP-1M3R group (79 pM) compared to the wild-type group (34 pM; median values) [46]. These results strongly highlight the importance of secretory capacity of transplanted islets for a successful outcome. This is particularly relevant when using islets from neonatal piglets that are easier and cheaper to isolate besides being capable of undergoing further maturation and proliferation *in vivo* after transplantation [12] and even *in vitro* when cultured on a collagen matrix [16]. Interestingly, we did not observe any deterioration of transgenic islet function throughout the 9-month follow-up period in contrast to what has been reported in patients

receiving sulfonylurea treatment to enhance their endogenous insulin production [50, 51]. Indeed, the InsGLP-1M3R modification acts by activating amplifying mechanisms without affecting the triggering pathway of insulin secretion whereas sulfonylureas directly bind K_{ATP} channels thus triggering insulin secretion. On the longer term, this is further supported by our observation that InsGLP-1M3R pigs maintain their glucose homeostasis and their improved insulin secretion from birth through adult age of up to 4 years. In conclusion, genetic modifications aiming to improve porcine islet insulin secretion such as the one discussed here can be as instrumental as immunology-oriented modifications in guaranteeing sufficient graft function to achieve normoglycemia.

AUTHOR CONTRIBUTIONS

NM and PG wrote the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GENERATIVE AI STATEMENT

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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REVIEW
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Current Techniques of Gene Editing in Pigs for Xenotransplantation

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Shortage of human organs for transplantation has created a demand for alternative solutions of which xenotransplantation is amongst the most promising one in the short term. However, the immune reaction following transplantation of a pig organ is greater than the one elicited during allotransplantation. Genetic engineering of the pig is required so that pig organs or tissues are made less immunogenic to humans by eliminating some antigens and by expressing human proteins that can reduce the damage by the host immune system. To generate founder animals with the desired mutations genetic engineering of somatic cells with multiplexed mutations combined with somatic cell nuclear transfer (SCNT) is the best solution with the technology available today. Safety concerns include potential zoonosis, primarily porcine endogenous retroviruses (PERVs). Ethical considerations might arise from the use animals involved in research. Genome editing techniques based CRISPR-Cas9, have greatly facilitated the modification of pig's genome to address coagulation and inflammation issues, to mention just a few, arising after the pig organ is transplanted into a human. However, further research is needed to ensure safety and efficacy of the genome edits introduced in the pig genome are compatible with the health and welfare of the pigs.

Keywords: pig, genetic engineering, CRISPR-Cas9, somatic cell nuclear transfer, xenotransplantation

INTRODUCTION

For many years, organ transplantation has been a critical life-saving option for individuals with end-stage organ failure. However, the gap between the need for organs and the available supply remains huge, resulting to long waiting lists, extended suffering, and preventable deaths. Xenotransplantation, which involves using organs from genetically modified animals, particularly pigs, offers a promising solution to this dilemma. Pigs are considered ideal donors due to their biological similarities to humans, compatibility in organ size, ease of breeding, genetic modification [1, 2], and the availability of gene editing tools [3, 4]. Additionally, pigs reproduce efficiently and have short generational intervals. Other livestock species are also being utilized as sources of biological materials for xenotransplantation or as bioreactors in biomedical applications [5–7]. Cattle, for example, are used to produce bioprosthetic heart valves from their pericardia [8]. Multiple immunological challenges have been identified (**Table 1**) and are being addressed through both genetic engineering and clinical immunosuppression protocols. Ensuring the safety of xenotransplantation is crucial, with particular concerns about the potential transmission of diseases, such as porcine endogenous retroviruses (PERVs) [9] and porcine cytomegalovirus (PCMV) [10]. Nevertheless, no cases of PERV transmission to humans following tissue xenotransplantation have been reported to date [11–13] and this threat has set back the field for

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TABLE 1 | Immunological barriers to xenotransplantation that can be abrogated through Genetic Engineering (adapted from Perota and Galli, 2016) [14].

Problem	Possible cause	Possible solution
Hyperacute rejection (HAR)	Pre-formed antibodies against Galactose α1-,3-galactose and other non-Gal antigens (Neu5Gc); activation of the complement cascade	KO of α1-3 galctosyltransferase, CMAH, B4GALNT2, iGb3S and other non-Gal antigens Expression of hCRP (CD55, CD46, CD59)
Acute humoral xenograft rejection (AHXR)	*De novo* antibodies against Galactose α1-,3-galactose and other non-Gal antigens (Neu5Gc); activation of the complement cascade. Endothelial cell activation; Thrombotic microangiopathy Consumptive coagulopathy	hTBM, hEPCR, hA20, TFPI, CD39,HMOX1
Immune cell-mediated rejection (ICMR)	NK and T-cell activation	hTRAIL, CTLA4Ig, HLA-E, huβ2m, CD47, SLA class I
Instant Blood-Mediated Inflammatory Reaction (IBMIR)	Surface proteins, complement mediated, innate immunity, platelets and leucocytes activation	All of the above genetic modifications

KO, Knock Out; Neu5Gc, N-Glycolylneuraminic acid; CMAH, CMP-N-acetylneuraminic acid hydroxylase; B4GALNT2, Beta-1,4-N-Acetyl-Galactosaminyl Transferase 2; iGb3S, isogloboside 3; hCRP, human complement regulatory proteins; hEPCR, human endothelial protein C receptor; TFPI, tissue factor pathway inhibitor; TRAIL, human tumor necrosis factor related apoptosis inducing ligand.

more than 20 years. Recently, genetically modified pig organs have been successfully transplanted into brain-dead human patients [15, 16], and for the first time, into living patients under compassionate use [17]. Xenotransplantation also raises ethical questions concerning the welfare of animals used in research and the broader impact on animal populations both on the pig side but also on the use of NHPs for pre-clinical studies. The development of somatic cell nuclear transfer (SCNT) [18] and advancements in genome editing tools like CRISPR-Cas9 have revolutionized the field, leading to rapid progress in the genetic engineering of pigs for transplantation purposes [19, 20]. Scientists aim to modify pig genomes to increase compatibility with human recipients, minimize organ rejection, and reduce the risk of disease transmission. Although genome editing for xenotransplantation remains in the research phase, another emerging area of exploration involves creating pig-human chimeric organs, which presents significant scientific and ethical challenges [21]. This approach, again based on genome editing and SCNT, entails generating defective pig embryos for a specific organ, followed by combining them with human pluripotent stem cells (PSCs) to allow the PSCs to develop the targeted organ [22, 23], however at present it is not possible to prevent human PSCc to colonize other organs of the pig fetus. This review will discuss the various steps and challenges involved in generating viable animals, from selecting the target gene to cloning and animal birth.

GENETIC ENGINEERING (GE) OF SOMATIC CELLS

In the past 2 decades, programmable nucleases have revolutionized genome editing, enabling precise alterations of the genetic code [19, 24–26]. Among these (see **Table 2**), CRISPR-Cas9 has become the most popular due to its simplicity, versatility, and low cost [27]. As the technology advances, more variants of CRISPR/Cas9 are expected to emerge. Effective application of these tools requires accurate DNA sequencing, along with software to assist in nuclease design, target site selection, and experimental validation, minimizing unintended effects known as off-target mutations [28–30]. SNPs present in different breeds or individuals might make inefficient the genome editing as the target site will not be recognized therefore it is important to sequence the target pig line to be used. These nucleases have been successfully used for gene editing across various species, including livestock, for both agricultural [31, 32] and biomedical [33, 34] purposes. In the biomedical field, pigs have long been a focus of genetic modification, particularly for xenotransplantation research. Typically, genetic editing in pigs target one [35, 36] or more specific loci, especially when inactivating endogenous genes through knockout (KO) methods. A well-known example is the genetic inactivation of the enzyme (GGTA1) responsible for the expression of the galactose α 1-3 galactose epitope (α-Gal) responsible for hyperacute rejection since is widely distributed on animal cell surfaces [37], followed by the KO of the enzyme CMAH, which is involved in producing the Neu5Gc antigen [36, 38–40] that is responsible for antibody mediated rejection. More recently, the simultaneous KO of GGTA1, CMAH, and B4GalNT2 (beta-1,4-N-acetyl-galactosaminyltransferase 2, also induces an antibody mediated response in humans) has been achieved [40, 41] and this genetic background is considered a basic requirement on where to build further gene edits. CRISPR-Cas9 has also been used to efficiently create multiple mutations in one round, for example, targeting three xenoantigens mentioned above simultaneously [40]. Another advancement in the CRISPR system involves the use of cytosine base editors (CBE), which can convert C to T without causing double-strand breaks (DSBs). This approach is used to silence endogenous genes by inducing nonsense mutations, offering a safer alternative to traditional methods like ZFNs or Cas9 [42, 43]. Since these modifications are made to cells cultured *in vitro*, researchers have ample opportunity to select cell clones with the precise mutation before using SCNT to generate the animals with the desired genotype. A simple and direct injection of CRISPR/Cas9 into zygotes can also produce genetically engineered animals [5, 44], this method though is less efficient when multiplexed genome editing is required and the risk of mosaicism, timepoint of microinjection is crucial to

TABLE 2 | Comparison of different programmable nuclease platforms used in livestock genome editing (adapted from [24] with permission from the Publisher).

	Zinc finger nuclease	TALEN	CRISPR/Cas9
Recognition site	Typically 9–18 bp per ZFN monomer, 18–36 bp per ZFN pair	Typically 14–20 bp per TALEN monomer, 28–40 bp per TALEN pair	22 bp (20-bp guide sequence + 2-bp protospacer adjacent motif (PAM) for *Streptococcus pyogenes* Cas9); up to 44 bp for double nicking
Specificity	Small number of positional mismatches tolerated	Small number of positional mismatches tolerated	Positional and multiple consecutive mismatches tolerated
Targeting constraints	Difficult to target non-G-rich sequences	5 targeted base must be a T for each TALEN monomer	Targeted sequence must precede a PAM
Ease of engineering	Difficult; may require substantial protein engineering	Moderate; requires complex molecular cloning methods	Easily re-targeted using standard cloning procedures and oligo synthesis
Immunogenicity	Likely low, as zinc fingers are based on human protein scaffold; FokI is derived from bacteria and may be immunogenic	Unknown; protein derived from *Xanthamonas* sp.	Unknown; protein derived from various bacterial species
Ease of *ex vivo* delivery	Relatively easy through methods such as electroporation and viral transduction	Relatively easy through methods such as electroporation and viral transduction	Relatively easy through methods such as electroporation and viral transduction
Ease of *in vivo* delivery	Relatively easy as small size of ZFN expression cassettes allows use in a variety of viral vectors	Difficult due to the large size of each TALEN and repetitive nature of DNA encoding TALENs, leading to unwanted recombination events when packaged into lentiviral vectors	Moderate: the commonly used Cas9 from *S. pyogenes* is large and may impose packaging problems for viral vectors such as AAV, but smaller orthologs exist
Ease of multiplexing	Low	Low	High

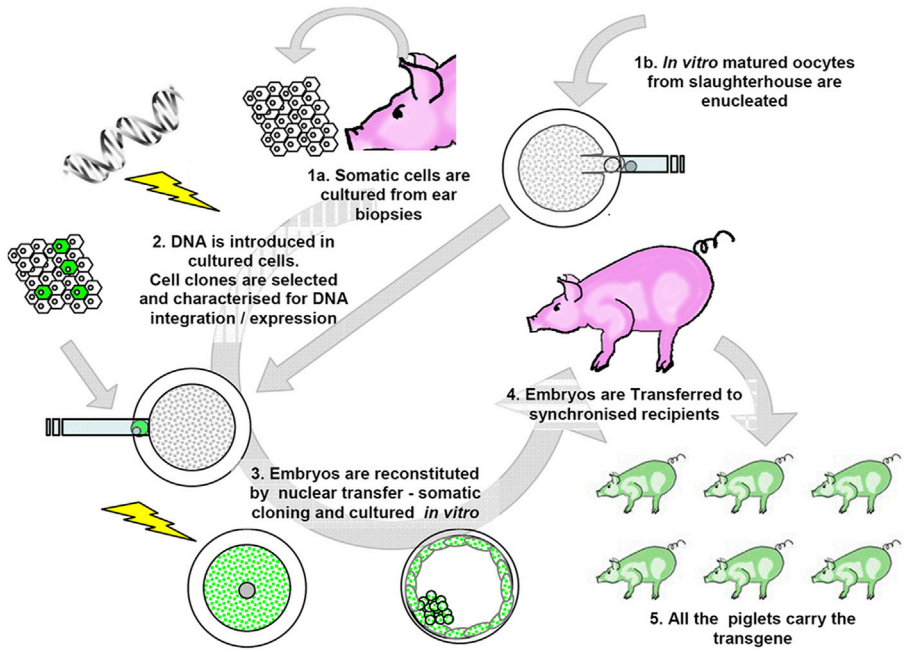


FIGURE 1 | Transgenic production by SCNT. The transgene is transferred into the genome of cultured fibroblasts. Transgenic cell clones are isolated and characterized. This first step is relatively inexpensive. A more accurate prediction of the transgene expression is possible. Next, embryos are reconstituted by SCNT, cultured, and transferred in synchronized sows. Although the viability of cloned embryos is variable but usually poor, all of the resulting newborns carry the gene edits of the donor cell [66].

avoid mosaicism. Mosaic animals, which result from genetic editing occurring at later stages of embryonic development (cleavage stage), may not carry the desired mutations in all cells including germ cells and as a consequence do not transmit them to their offspring [45]. This risk is particularly concerning in livestock species with long generation intervals, making SCNT from validated cell clones a more reliable method ensuring that all the animals are carrying the exact mutation and will transmit to their progeny despite its low efficiency. In xenotransplantation, certain "safe harbor' loci can be targeted for KO of xenoantigens like GGTA1 [46] or CMAH, allowing for single-copy gene integration without disrupting other genes and at the same time ensuring expression of the transgene introduced. Additionally, specific

solutions [47] could be used to address potential lethal effects of certain transgenes during embryonic development or early after birth. More advanced technologies may, in the future, help further control gene functionality. For example, if a transgene requires tissue-specific expression, such as in endothelial cells [48] or in insulin-producing cells [49], this can help minimize side effects of overexpression in all tissues that might impact homeostasis of the animal and ensure that genetic engineering (GE) remains compatible with the animal's survival. Another method to control transgene expression is the use of inducible promoters, that can be activated by administering to the pregnant sows or after birth of the piglets, substances like tetracycline [50] or doxycycline [47]. This allows transgene expression to be switched on when needed, either during the animal's life or after the organ is transplanted into the patient. Disadvantage of on-systems in the patient is a potential lifelong necessary administration of antibiotics. A third approach involves RNA interference (RNAi) technology, which has been used to reduce the expression of porcine endogenous retroviruses (PERVs) [51, 52], as they exist in multiple copies within the porcine genome, or to downregulate the expression of pig Tissue Factor [53], where a full gene knockout would be lethal. In such cases, small interfering RNA (siRNA) is highly effective, capable of reducing gene expression by 95% or more, though it does not completely eliminate the gene's activity. One long-term issue with using commercially bred pigs for xenotransplantation is the continued growth of the organs after transplantation, potentially causing complications for the recipient [54]. This issue is relevant mainly for heart while kidney, for example, being in the abdominal cavity can tolerate the growth. One approach attempted to reduce size of the pig is to genome edit farm pigs to knock out the growth hormone (GH) receptor. However, with this size reduction, the pigs grow up to 60% of their normal size, also leads to unwanted health issues in the pigs since this mutation is responsible of a genetic disease making the breeding of these pigs not sustainable in the long term [55, 56]. Possible solutions already in development include using smaller breeds [57] or minipigs [58].

FROM GENOME EDITED CELLS TO ANIMALS

Selecting the Cell Line

The choice of the cell line is a crucial factor in the success of Somatic Cell Nuclear Transfer (SCNT) embryo production and remains one of the key variables that can determine success or failure. Factors such as culture conditions, cell doubling rates, and oxygen levels [59] can influence which cell populations or sub-populations emerge during *in vitro* culture, affecting chromatin status and, most importantly, the ability of cells to be reprogrammed following nuclear transfer. Identifying cell lines with high SCNT efficiency can produce outstanding results, such as 10% livebirths on average being 2%–5% on the number of embryos transferred, whereas others may lead to significant failures [60].

For cloning purposes, skin fibroblasts from biopsies are the most used cell type when the genotype or phenotype of the donor animal is already known and must be perpetuated. However, when this is not the case, fetal fibroblasts are typically preferred, especially for genetic engineering (GE) applications. Some investigators also prefer to harvest early stage fetuses of 25–30 days of gestation [61, 62] if new edits must be added to that genetic background. While many studies debate the most efficient cell types for pig cloning [63, 64], these recommendations may sometimes conflict with the specific requirements of certain projects. In general, GE of the cell line used for SCNT does not significantly reduce its effectiveness in producing viable offspring, though a slight decrease in efficiency has been noted in gene knockout (KO) experiments [63, 64].

All cell lines can be cryopreserved at early passages before genetic modification, ensuring that the same cell lines can be reused in multiple rounds of nuclear transfer, thus controlling a critical variable in the SCNT process.

Embryo Production

Over the years, the basic principles of cloning through nuclear transfer in livestock have remained consistent with the methods pioneered by Willadsen [65] and later adapted for somatic cells [18]. The process begins with the preparation of a matured enucleated oocyte, in which the metaphase plate together with the polar body is removed through micromanipulation. Next, a nucleus from a somatic cell carrying the desired genetic modifications is transferred into the enucleated oocyte by cell fusion by positioning the somatic cell into the perivitelline space or by adhering it with phytohemagglutinin in the case of zone free SCNT. Finally, the oocyte is activated either chemically or electrically to resume the cell cycle. The resulting embryos are then either transferred at the one-cell stage to the oviducts of synchronized recipient animals or cultured to the blastocyst stage before being transferred to the uterus of the recipient gilt (see **Figure 1**) [66]. A large number of metaphase II oocytes, necessary for embryo production, can be sourced inexpensively from slaughterhouses, adhering to the 3R principles (Replacement, Reduction, and Refinement). Procedures for oocyte maturation and embryo culture are well established in pigs [67] and are applied similarly in SCNT. However, micromanipulation remains a bottleneck in the process, requiring specialized equipment and skilled embryologists, as it is labor-intensive. Visualization of the metaphase plate is usually achieved using Hoechst staining and UV light, as livestock oocytes are rich in lipids, making them darker than those of mice or humans. Despite their normal morphology, SCNT embryos in the pre-implantation stage have reduced potential to develop to term [68]. This limitation is tied to the "black box' of cellular reprogramming, how the nucleus of the donor cell is reset to support normal embryo development. Currently, this process remains inefficient and is still poorly understood [69, 70] in livestock species, although some progress has been made in mice [71]. In mice, the use of Trichostatin A (TSA), a histone deacetylase inhibitor, during the early hours of culture after nuclear transfer has shown significant improvements in live birth rates by promoting chromatin demethylation and enhancing reprogramming [72]. Similar strategies, involving various demethylating agents, have shown promising results in pigs in some laboratories [73].

Pregnancy

The ability of SCNT embryos to establish pregnancies is generally lower than that of embryos created through fertilization. This discrepancy has economic implications, as it increases the cost of maintaining recipient animals that are either not pregnant or that experience pregnancy loss. In pigs, this issue can be partially mitigated by transferring a large number of embryos, as SCNT embryo production is typically not a limiting factor. Pigs can tolerate the transfer of over 100 embryos, adjusting naturally through physiological reabsorption of excess embryos. Another challenge with SCNT pregnancies is that they often have a prolonged gestation period, frequently necessitating induced parturition or cesarean sections because of the presence of a small number of fetuses or fetuses of small size [74].

Offspring

The success rate of SCNT in terms of development to term can reach up to 16% [75], depending on whether the rate is calculated based on the number of reconstructed embryos or transferred blastocysts. However, various factors, such as pig breed and the cell line used for genetic modification, can affect this success rate, making comparisons difficult [64, 76]. SCNT offspring are more fragile at birth and have higher stillborn and perinatal mortality rates. To improve their survival, special attention is required during farrowing and neonatal care in the first week of life. Once they survive the critical first days or weeks, cloned animals generally live normal lives, are fertile, and do not pass on any phenotypical abnormalities to their offspring [77–80]. An important consideration for the broader application of this technology is the impact of genome editing on the viability of the animal and its ability to breed naturally. It's crucial to determine how many genome edits are necessary and compatible with maintaining the animal's homeostasis [81, 82], as well as whether the inserted transgenes are expressed at the desired levels. This requires thorough genotyping and phenotyping of newborn animals. A systematic approach might be necessary to assess each genetic modification before proceeding further. This should also be confirmed in the F1 generation (F0 are considered the founder animals) to be sure that the expression of the transgenes is maintained while breeding the animals by sexual reproduction. Once the pig line is established, to be sustainable from an economic point of view and for health and welfare reasons for the animals conventional breeding should be the priority. One example of potentially unnecessary genetic modification is the inactivation of all 64 copies of porcine endogenous retroviruses (PERVs) in a pig line [9], even though there has never been documented transmission of PERVs to humans in previous xenotransplantation experiments [11–13]. Additionally, PERVs may have an as-yet-undiscovered physiological role in the genome, which warrants further studies [83, 84].

CONCLUSION

Remarkable progress has been made in the genetic engineering of pigs in general and specifically to produce organs suitable for transplantation to humans. This has lead to the first pig to human transplantation of heart in living patients under compassionate use [17, 85] and in 2024–2025 the xenotransplantation of kidneys into clinical patients [86] at the same time FDA has given approval for the first IND application to initiate clinical trials [87]. Gene-editing technologies, particularly CRISPR-Cas9, have been employed to modify pig genes associated with immune rejection, viral transmission, and compatibility issues, leading to the development of pigs with reduced immunological challenges and increased suitability for human recipients. Genome editing technologies are continuously developed to make them more effective and adaptable to the different need of gene editing while reducing potential side effects [88, 89]. However, one of the primary obstacles in xenotransplantation remains the immune response triggered by pig organs in humans, which results in organ rejection. Genetic engineering is focused on overcoming this barrier by altering or removing problematic genetic elements. Although substantial advancements have been achieved, further research is essential to ensure long-term graft success and avoid immune-driven rejection. The genetic modification of pigs also raises ethical questions, particularly regarding animal welfare and the broader implications of genome alterations in animals. It is vital to ensure the wellbeing of genetically modified pigs, guided by strict ethical standards and practices. As a highly regulated and complex field, xenotransplantation requires extensive preclinical research, safety evaluations, and approval from regulatory bodies before it can become a standard medical procedure. Clinical trials will be necessary to assess the safety and effectiveness of pig organ transplantation in humans and a number of non-human primates will also be required for that. In summary, genetically engineering pigs for xenotransplantation holds great promise in addressing the global organ shortage. While considerable strides have been made, more research is needed to overcome immunological challenges, reduce the risk of pathogen transmission, address ethical concerns, and meet regulatory and clinical requirements. Future scientific developments, combined with rigorous safety protocols and ethical considerations, will be pivotal in successfully translating pig genetic engineering into viable and safe xenotransplantation therapies while preserving the health and welfare of the animals involved.

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The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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What Genetic Modifications of Source Pigs Are Essential and Sufficient for Cell, Tissue, and Organ Xenotransplantation?

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Xenotransplantation of porcine organs has made remarkable progress towards clinical application. A key factor has been the generation of genetically multi-modified source pigs for xenotransplants, protected against immune rejection and coagulation dysregulation. While efficient gene editing tools and multi-cistronic expression cassettes facilitate sophisticated and complex genetic modifications with multiple gene knockouts and protective transgenes, an increasing number of independently segregating genetic units complicates the breeding of the source pigs. Therefore, an optimal combination of essential genetic modifications may be preferable to extensive editing of the source pigs. Here, we discuss the prioritization of genetic modifications to achieve long-term survival and function of xenotransplants and summarise the genotypes that have been most successful for xenogeneic heart, kidney, and islet transplantation. Specific emphasis is given to the choice of the breed/genetic background of the source pigs. Moreover, multimodal deep phenotyping of porcine organs after xenotransplantation into human decedents will be discussed as a strategy for selecting essential genetic modifications of the source pigs. In addition to germ-line gene editing, some of these modifications may also be induced during organ preservation/perfusion, as demonstrated recently by the successful knockdown of swine leukocyte antigens in porcine lungs during *ex vivo* perfusion.

Keywords: pig, genetic modification, xeno-antigens, complement activation, coagulation dysregulation

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INTRODUCTION

Despite their large phylogenetic distance from humans, pigs have become the preferred species as a source of cells, tissues, and organs for xenotransplantation. Major reasons include their favorable reproductive biology (a gestation period of less than 4 months, multiple offspring, sexual maturity at 5–8 months), their propagation in designated pathogen-free (DPF) facilities, the ethically accepted use of pigs for medical purposes, and an established toolkit for efficient and precise genetic modification [1]. The latter is necessary because pigs are a discordant organ source for humans in several respects. Most importantly, porcine cells carry carbohydrate antigens, such as galactose-α1,3-galactose (αGal),

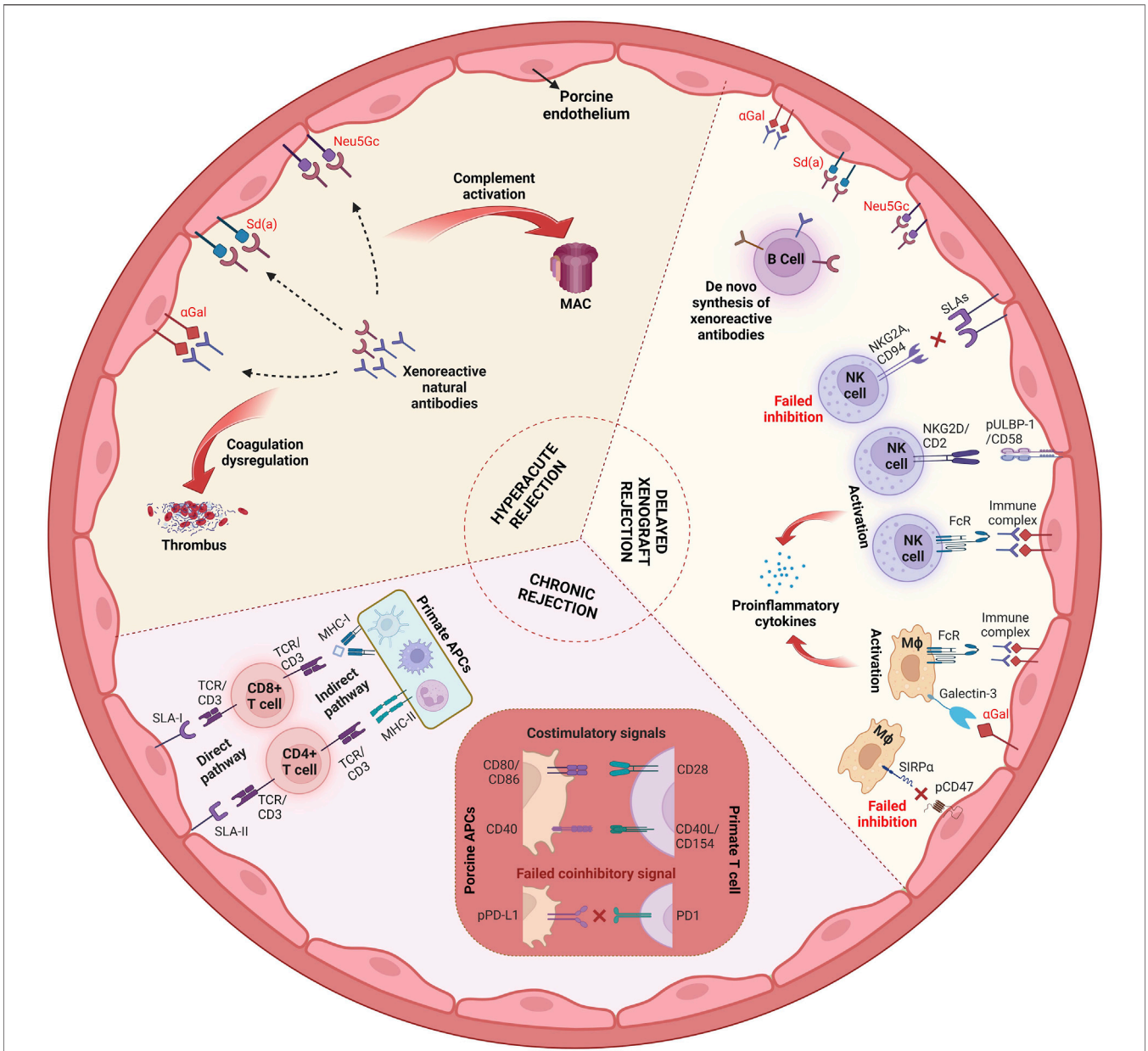


FIGURE 1 | Mechanisms of xenograft rejection. Abbreviations: MAC, membrane attack complex; NKG2A, inhibitory NK receptor; NKG2D, activating NK receptor; pULBP-1, porcine UL-16-binding protein; FcR, Fc receptor; SLA, swine leukocyte antigen; Mϕ, macrophage; PD1, programmed cell death protein 1; pPD-L1, porcine programmed death ligand-1; APC, antigen-presenting cell; MHC, major histocompatibility complex; TCR, T cell receptor (Created with BioRender.com).

N-glycolylneuraminic acid (Neu5Gc), and an Sda-like blood group antigen, against which humans and, in part, also non-human primates (NHPs) have pre-formed natural antibodies (pnAbs). Upon xenotransplantation of porcine tissues or organs into primates, the pnAbs bind to their carbohydrate antigen targets, inducing activation of the complement system, endothelial cell activation, coagulation, and antibody-dependent cellular cytotoxicity (ADCC). Therefore, wild-type porcine xenotransplants are hyperacutely rejected upon transplantation into humans and non-human primates [2].

This can be addressed by generating source pigs lacking the major carbohydrate antigens and expressing one or several human complement pathway regulatory proteins (see below). However, coagulation dysregulation can still occur after xenogeneic organ transplantation due to incompatibilities between membrane-bound factors on porcine endothelial cells and soluble components in human/NHP blood [3]. An example is the thrombin-thrombomodulin (THBD) interaction. Within a species, THBD on the endothelial cells binds thrombin from the circulation, and – supported by an endothelial protein C receptor

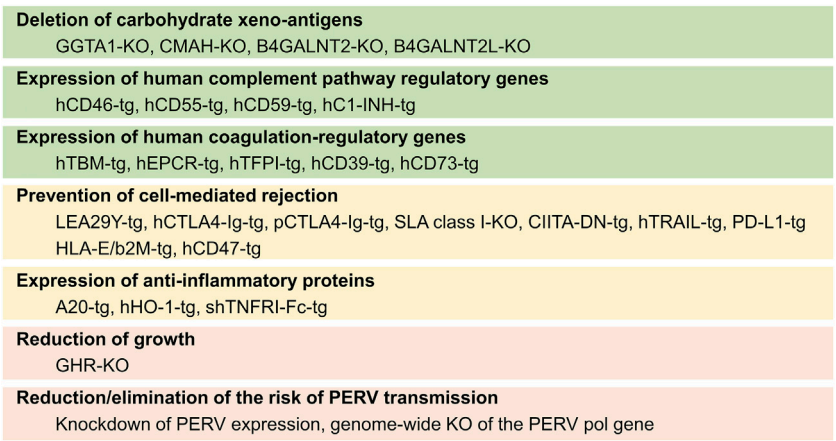


FIGURE 2 | Summary of genetic modifications in source pigs for xenotransplantation and the goals for which they are introduced. KO, knockout/inactivation; tg, transgenic.

(EPCR) – the THBD-thrombin complex activates protein C. Activated protein C has an anti-coagulation effect. In a pig-to-primate organ xenotransplant, the porcine endothelium is exposed to human/NHP blood. Porcine THBD can bind human/NHP thrombin, but the complex is inefficient in the activation of protein C, leading to coagulation in the small blood vessels and thrombotic microangiopathy. Thus, adaptations of the source pigs are mandatory to avoid coagulation dysregulation in xenotransplanted organs (see below).

In addition, porcine xenotransplants undergo cell-mediated rejection as they trigger, or fail to prevent, the activation of innate immune cells, such as natural killer (NK) cells and macrophages, as well as the adaptive cellular immune system (T and B cells). As swine leukocyte antigen (SLA)-I cannot effectively bind primate inhibitory NK cell receptors, there is, in addition to ADCC, direct human NK cell cytotoxicity against porcine cells. Moreover, macrophages are activated by porcine cells because porcine CD47 does not bind the "do not eat me" signal regulatory protein alpha (SIRPα) on human macrophages. Activation of human or NHP T cells against porcine xenotransplants occurs either directly via the presentation of porcine peptides by porcine antigen-presenting cells (APCs) or indirectly via human or NHP APCs. Several costimulatory [most importantly CD40—CD40L (CD154) and CD80/86—CD28] and coinhibitory signals (PD-L1—PD-1) are involved in this process. B cells contribute to xenograft rejection by producing antibodies that target the graft, leading to complement activation, ADCC, and chronic immune responses that result in graft rejection. **Figure 1** provides an overview of the mechanisms involved in xenograft rejection.

GENETIC MODIFICATION OF SOURCE PIGS FOR CELL, TISSUE, AND ORGAN TRANSPLANTATION

The use of genetically modified (GM) source pigs for cell, tissue, and organ transplantation has been extensively reviewed recently [4, 5]. The most prominent modifications, in particular, those tested in transplantation studies in NHPs or even human brain-dead or live patients are shown in **Figure 2**.

There is broad consensus that the elimination of the primary xeno-antigens (αGal, Neu5Gc, and Sda) is mandatory to prevent the binding of preformed natural primate anti-pig antibodies. This is achieved by introducing loss-of-function mutations of the α-1,3-galactosyltransferase (*GGTA1*), cytidine monophosphate-N-acetylneuraminic acid hydroxylase (*CMAH*), and β-1,4-N-acetyl-galactosaminyl transferase 2 (*B4GALNT2*)/B4GALNT2-like (*B4GALNT2L*) genes, thus producing triple knockout (TKO) pigs. To minimize complement-mediated injury to the xenotransplant, which can also be triggered by ischemia-reperfusion injury or by the binding of antibodies produced *de novo* against porcine antigens, the transgenic expression of one or several human complement pathway regulatory proteins (CPRPs), such as CD46, CD55, and CD59, is often attempted (**Figure 2**).

Coagulation dysregulation has been overcome by transgenic expression of human THBD in the source pigs. While porcine EPCR appears to be functionally compatible with the human protein C pathway, transgenic overexpression of human EPCR is expected to enhance protective thromboregulation. Other genetic modifications targeting coagulation dysregulation include transgenes for human tissue factor (TF) pathway inhibitor (TFPI), human ectonucleoside triphosphate diphosphohydrolase 1 (CD39), 5′-nucleotidase ecto (NT5E, CD73), or the siRNA-mediated knockdown of porcine TF expression.

Transgenic expression of human leukocyte antigen (HLA)-E/beta2-microglobulin (B2M) is a strategy to inhibit the activation of human NK cells carrying the inhibitory receptor CD94/NKG2A, while the activation of human macrophages can be inhibited by the expression of human CD47. Transgenes for CTLA4-Ig or its higher-affinity derivative LEA29Y have been developed to block the CD28—CD80/CD86 co-stimulatory pathway of T cell activation. Alternatively, the negative

coregulatory PD-L1—PD-1 pathway was employed by transgenic expression of human PD-L1. Pigs lacking or expressing reduced levels of SLA class I have been achieved by knockout or knockdown strategies of B2M, while the expression of SLA class II was reduced by transgenic expression of a human dominant-negative mutant class II transactivator (CIITA-DN).

In addition, transgenic pigs expressing anti-inflammatory proteins such as human TNF-alpha-induced protein 3 (TNFAIP3 alias A20), human heme oxygenase 1 (HMOX1), or soluble human tumor necrosis factor receptor I IgG (1)-Fc (shTNFRI-Fc) have been produced to diminish inflammation escaping control by other genetic modifications (reviewed in [3]).

As a measure to prevent excessive growth of the source pigs and their organs, the knockout of the growth hormone receptor (*GHR*) gene has been proposed [6]. This modification reduces the size of the pigs to about 50% of the wild type, but also causes metabolic changes and leads to obesity [7, 8]. Therefore, it is preferable to use an originally smaller genetic background that fits the size of human recipients. Examples are Yucatan miniature pigs or Auckland Island pigs. The latter are characterized by a high level of genetic homogeneity without signs of inbreeding depression, including a uniform SLA makeup, which facilitates tolerance induction strategies. Moreover, Auckland Island pigs have excellent heart function and appear free of cardiac malformations [9], while Yucatan miniature pigs display an increased rate of ventricular septum defects [10, 11].

Modifications aiming to reduce or eliminate the risk of porcine endogenous retrovirus (PERV) transmission include the knockdown of PERV expression or the genome-wide mutagenesis of the PERV *pol* gene by CRISPR/Cas9 genome editing [12]. Alternative strategies include antiviral treatment and vaccination of the recipient against PERV [13].

GM COMBINATIONS FOR XENOGENEIC HEART TRANSPLANTATION

As of July 2024, around 1,034 patients were on the waiting list for a heart transplant within the Eurotransplant region, with the global numbers being much higher [14]. We recently published a comprehensive review on the use of GM pigs as donors in cardiac xenotransplantation [15]. Key factors for achieving long-term survival include protecting the xenograft from the host immune response, ensuring nonischemic preservation of the xenograft before implantation, developing a clinically applicable immunosuppressive regimen, and managing post-implantation xenograft growth [15]. By knocking out (KO) the *GGTA1*, *CMAH*, and *B4GALNT2/B4GALNT2L* genes to eliminate xenoantigens in donor pigs, and introducing transgenic expression of human complement and/or coagulation regulatory factors, the host immune response is significantly reduced [16].

Numerous pig-to-NHP preclinical trials have been conducted to determine the optimal combination of genetic modifications for successful cardiac xenotransplantation [4]. In 2014, Mohiuddin et al. heterotopically transplanted a 3-GM pig heart with *GGTA1*-KO and transgenic expression of human CD46 and THBD into an immunosuppressed baboon and achieved a long-term survival of over 200 days [17]. Building on this success, in 2016, they transplanted in the same heterotopic setting similar 3-GM pig hearts into baboons along with a modified immunosuppressive regimen and achieved survival of up to 2.5 years (**Figure 3**) [18]. Recently, Chaban et al. [19] performed heterotopic transplantations of hearts from 3-GM (*GGTA1*-KO, *B4GALNT2*-KO, and human CD55 expression), 9-GM (KO of *GGTA1*, *B4GALNT2*, and GHR, along with human CD46, CD55, THBD, EPCR, CD47, and HMOX1 expression), or 10-GM (9-GM plus *CMAH*-KO) donor pigs into immunosuppressed baboons. In this study, a maximum survival time of 393 days was achieved in a 9-GM pig heart recipient [19]. In 2018, the first successful series of life-supporting orthotopic pig-to-NHP heart transplantations was achieved by Längin et al. [20]. They transplanted 3-GM pig hearts with *GGTA1*-KO and transgenic expression of human CD46 and THBD into baboons under standard immunosuppression and achieved a maximum survival of 195 days (**Figure 3**) [20]. Importantly, in these preclinical trials, post-implantation heart growth was regulated by lowering the recipient's blood pressure to match the porcine level, regulating cortisone levels, and using the sirolimus prodrug, temsirolimus, to reduce myocardial hypertrophy [20]. In the same year, Hinrichs et al. introduced a novel approach for managing post-transplant heart growth by KO of the *GHR* gene in donor pigs [7]. This genetic modification was first tested by Goerlich et al. in 2021, who orthotopically transplanted 7-GM pig hearts with KOs of *GGTA1*, *B4GALNT2*, and *GHR*, along with transgenic expression of human CD46, THBD, EPCR, and CD47 into immunosuppressed baboons, and achieved survival of up to 264 days [21]. In 2022, Mohiuddin et al. [22] orthotopically transplanted hearts from pigs with 3–9 genetic modifications into baboons. The longest survival time recorded was 264 days in a baboon that received a 7-GM heart, specifically with the KO of *GGTA1*, *B4GALNT2*, and *GHR*, along with human CD46, THBD, EPCR, and CD47 expression [22].

The first pig-to-human life-supporting heart transplantation took place on 7 January 2022, when a 57-year-old man received a 10-GM pig heart from Revivicor. This heart was from a cloned pig and had KOs of *GGTA1, CMAH, B4GALNT2/B4GALNT2L*, and *GHR*, and carried expression cassettes for human CD46, CD55, THBD, EPCR, CD47, and HMOX1 (**Figure 3**) [23]. The xenograft functioned normally for 49 days post-transplant before experiencing signs of rejection such as sudden myocardial thickening and xenograft failure, leading to the patient's death on day 60. It was reported that factors such as antibody-mediated rejection, complement-dependent cytotoxicity, and PCMV transmission from the xenograft might have contributed to the xenograft failure [23]. Later on 20 September 2023, a similar 10-GM heart was transplanted in a 58-year-old man, who lived for nearly 6 weeks post-transplant and died on 30 October 2023 [24]. Although the details about the clinical course of this patient are yet to be published, the heart showed signs of rejection days before his passing [24].

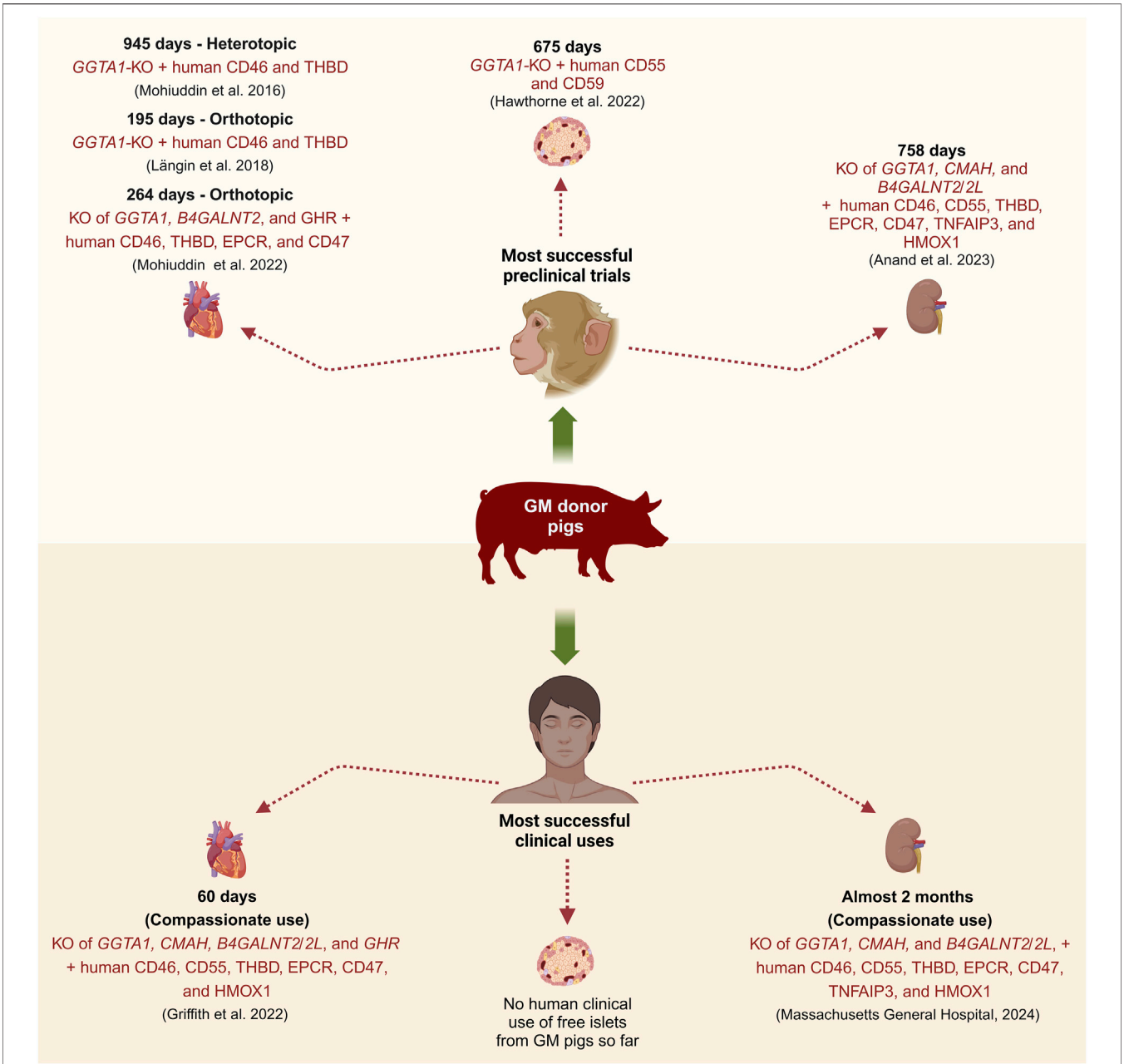


FIGURE 3 | Most successful preclinical and clinical transplantations of GM pig organs or tissue (Created with BioRender.com).

GM COMBINATIONS FOR XENOGENEIC KIDNEY TRANSPLANTATION

Approximately 1.2 million deaths each year are linked to end-stage renal disease (ESRD) [25, 26], and kidney transplantation remains the most effective treatment option [27–30]. The number of patients waiting for a kidney transplant is strikingly high, with over 10,000 patients waiting in the Eurotransplant region as of July 2024 [14]. Due to comparable sizes and similar metabolic and physiological functions of human and pig kidneys [31], pig-to-human kidney xenotransplantation can be useful to address the organ shortage. However, genetic modifications in donor pigs are required to circumvent certain immunological barriers in pig-to-human kidney xenotransplantation [12, 32].

Multiple research groups have achieved long-term survival of porcine kidney xenograft recipients in NHP preclinical trials [33–37]. Anand et al. and Ma et al. reported survival of up to 758 days using 10-GM pig kidneys, which were modified to KO *GGTA1*, *CMAH*, and *B4GALNT2/B4GALNT2L*, and express human transgenes such as CD46, CD55, THBD, EPCR, CD47, TNFAIP3, and HMOX1 along with retroviral (PERV) inactivation (RI), hence also claimed as 69-GM pigs

(**Figure 3**) [35, 37]. Similarly, Kim et al. and Adams et al. achieved survival of up to 557 days using double KO (*GGTA1* and *B4GALNT2/B4GALNT2L*) and triple KO (*GGTA1*, *B4GALNT2/B4GALNT2L*, and *SLA-I*) pigs, with or without human CD55 transgene [33, 34, 38]. Eisenson et al. reported a maximum survival time of 337 days, using the above-described 10-GM pigs from Revivicor [39]. The insights gained from these studies have paved the way for clinical trials.

In 2022, Montgomery et al. transplanted thymo-kidneys from *GGTA1*-KO pigs into two brain-dead human recipients, in the presence of native kidneys [40]. The study continued for 54 h and a significant increase in urine production and a reduction in creatinine level was observed. Although the initial analysis did not show any signs of hyperacute or antibody-mediated rejection [40], subsequent multimodal profiling revealed an antibody-mediated reaction, with a preference for the glomerular compartment [41]. Locke et al. performed the first clinical-grade trial and transplanted kidneys from the 10-GM Revivicor pigs into two human decedents who first underwent bilateral native nephrectomy [42–44]. The observation period was 74 h in the first and 7 days in the second recipient. In the first study, prominent thrombotic microangiopathy was observed without evidence of cellular rejection or antibody deposition, accompanied by low urine output and high creatine levels [42]. In the second study, the creatine clearance was improved over time, in the absence of thrombotic microangiopathy and some evidence of antibody-mediated rejection [43, 44].

The first compassionate porcine kidney xenotransplantation was done by transplanting a 69-GM pig kidney (3KO.7TG.RI) in an ESRD patient at Massachusetts General Hospital, who unfortunately died 2 months post-transplant due to cardiac arrest with a functioning kidney (**Figure 3**) [45]. The second patient at New York University suffered from heart and kidney failure, and received a left ventricular assist device (LVAD) on 4th April 2024, followed by thymo-kidney transplantation from a *GGTA1*-KO pig on 12th April 2024 [46]. The kidney xenograft failed after 6 weeks leading to its removal on 29th May 2024 [46].

GM COMBINATIONS FOR XENOGENEIC ISLET TRANSPLANTATION

For patients with type 1 diabetes, β-cell replacement is a superior therapeutic option over daily insulin injections [47]. Xenotransplantation of pig pancreatic islets is a viable alternative to allotransplantation, as discussed in several reviews [5, 48, 49]. Although the isolation of adult porcine islets (APIs) is challenging, some researchers prefer them over neonatal/fetal porcine islets (NPIs) due to their lower α-Gal expression, greater insulin production capacity, and immediate functionality post-transplantation [50]. In contrast, NPIs are easier to isolate but require functional maturation *in vitro*. Nevertheless, many research groups opt for NPIs from the 1- to 5-day-old pancreas and have developed concepts to improve their *in vitro* maturation [51–54].

Regardless of the islet source, over 50% of islets transplanted into the portal vein, a standard protocol in clinics, are lost due to the instant blood-mediated inflammatory reaction (IBMIR) [55]. This innate immune response is more severe for xenoislets than for alloislets [55, 56]. Therefore, overcoming the host immune response is crucial for successful islet xenotransplantation. In pig-to-NHP preclinical trials, surprisingly, the longest islet survival of over 900 days was achieved using WT APIs [57]. However, the recipients required a clinically unacceptable immunosuppressive regimen, and the engrafted pig islets were rejected once the immunosuppression was withdrawn [57]. Genetically modifying the donor pigs can help circumvent the host immune response. APIs from human CD46-expressing pigs survived for over 396 days in immunosuppressed diabetic cynomolgus monkeys [58], whereas NPIs from *GGTA1*-KO pigs survived for up to 249 days in immunosuppressed diabetic rhesus monkeys [59].

Hawthorne et al. [60] transplanted NPIs from 4-GM pigs with *GGTA1*-KO and transgenic expression of human CD55, CD59, and α1,2-fucosyltransferase (HT), into immunosuppressed non-diabetic baboons. Although these NPIs provoked minimal IBMIR, they were rejected 1 month after transplant due to cell-mediated rejection [60]. Bottino et al. [61] transplanted APIs from either 4-GM pigs (*GGTA1*-KO, and transgenic expression of human CD46, TFPI, and CTLA4-Ig), 5-GM pigs (*GGTA1*-KO, and transgenic expression of human CD46, CD39, TFPI, and CTLA4-Ig), or 1-GM pigs (expressing human CD46) into immunosuppressed diabetic cynomolgus monkeys. APIs from both 4- and 5-GM pigs evaded early IBMIR and survived for over 90 days [61]. Recently, Hawthorne et al. [62] transplanted NPIs from 3-GM pigs (*GGTA1*-KO and transgenic expression of CD55 and CD59) in diabetic baboons under judicious immunosuppression and achieved a survival of up to 675 days (**Figure 3**). A variable survival time of xenoislets in the preclinical trials suggests that optimal combinations of genetic modifications and clinically acceptable immunosuppression regimens are yet to be found.

Although several clinical trials have been conducted using encapsulated WT or GM porcine islets (reviewed in [5]), the clinical trials using free porcine islets are very limited. In the 1990s, Groth et al. [63] intraportally transplanted WT NPIs into a series of diabetic patients under standard immunosuppression. Although complete insulin independence was not achieved, porcine C-peptide was detectable in the urine of four patients for 200–400 days post-transplantation [63]. In 2011, Wang et al. injected WT NPIs in the hepatic artery of 22 diabetic patients under an immunosuppressive regimen [64]. Daily insulin requirement was reduced in 14 patients, and six patients were followed up for 4–6 years after the transplantation [64].

MULTIMODAL DEEP PHENOTYPING TO SELECT ESSENTIAL GENETIC MODIFICATIONS OF SOURCE PIGS

A possibility of evidence-based refinement of genetic modification of source pigs for xenotransplantation is the analysis of porcine organs that have been transplanted into

brain-dead or live patients. In a recent study, Loupy et al. [41] performed a multimodal phenotyping of two *GGTA1*-KO xeno-kidneys that had been transplanted into two brain-dead human recipients and maintained their circulatory and respiratory activity for 54 h [40]. The complex screening protocol combined histopathology, immunophenotyping (IgM, IgG, C4d, CD68, CD15, NKp46, CD3, CD20, and von Willebrand factor), bulk gene expression profiling, and whole-transcriptome digital spatial profiling, including cell deconvolution, to gain insights into spatially resolved immune reactions to the xenografts after 54 h. The cellular and molecular findings of the study indicated an antibody-mediated reaction preferentially to the glomerular compartment of the xeno-kidneys, associated with endothelial activation and the recruitment and activation of monocytes, macrophages, and natural killer cells, but with no evidence for complement activation. The study also highlighted the potential benefits of GM strategies of source pigs to inhibit the activation of macrophages (such as the expression of human CD47 [65]) and natural killer cells (e.g., expression of HLA-E/B2M [66]). The complex multimodal screening approach as introduced by Loupy et al. [41] has the potential to unravel hitherto unknown reactions to xenotransplants and has recently also been applied to two cases of cardiac xenografts from 10-GM pigs with KOs of *GGTA1*, *CMAH*, *B4GALNT2/B4GALNT2L*, and *GHR* and transgenic expression of human CD46, CD55, CD59, THBD, EPCR, and HMOX1 in brain-dead recipients [67, 68].

In contrast to life-supporting xenogeneic heart transplantation experiments in baboons [20, 69] and the first compassionate use transplantations in live patients [23], perfusion preservation of the porcine hearts was not performed in these studies in decedents [67]. Furthermore, standard immunosuppression plus the complement inhibitor eculizumab, but not CD40-CD154 co-stimulation blockade was used. While in both cases the xeno-heart was fully functional immediately after transplantation, the function declined in one case due to organ size mismatch and associated tissue hypoperfusion. The second heart was better size-matched and performed well throughout the study. The multimodal screening [68] of the hearts revealed corresponding molecular changes: an early immune response driven by T cell and natural killer cell activity and disruptions in cellular metabolism in the first heart, and only relatively minor changes in RNA, protein, lipid, and metabolic profiles in the second case.

Although the conclusions of these multimodal phenotyping studies are limited by the small sample size and short study duration [41, 68], the general strategy may provide new insights into human anti-porcine xeno-organ responses. This approach could also be applied to xenotransplantation studies in nonhuman primates where larger series with long-term outcomes exist [20, 22, 37, 39, 69].

GENETIC MODIFICATION OF THE ORGAN VS. THE SOURCE PIG

An interesting concept is the modification of organs for transplantation during preservation perfusion. Recently, Figueiredo and colleagues [70] explored this concept in a porcine allogeneic lung transplantation model. Donor lungs were lentivirally transduced during *ex vivo* perfusion to express short hairpin RNAs targeting β2-microglobulin (B2M) and class II transactivator mRNAs to knock down SLA expression. While all grafts in the control group with unmodified SLA expression (n = 7) were rejected within 3 months, five of the seven animals in the SLA-knockdown group maintained graft survival without immunosuppression during the 2-year monitoring period, demonstrating a clear survival advantage of the SLA-silenced organs [70]. The reduction of SLAs may be also important for xenotransplantation since late failure of pig-to-rhesus renal xenografts was associated with an increase in anti-swine leukocyte antigen class I and class II, particularly anti-SLA-DQ, antibodies [71].

CONCLUSION AND OUTLOOK

Defining what constitutes "sufficient genetic modifications for successful xenotransplantation" is one of the most frequently asked questions in the field and is equally challenging to address. Numerous research groups have made significant progress in identifying the key genetic modifications to evade the host immune response and ensure long-term xenograft survival. While various GM combinations have been tested *in vitro* and in preclinical trials, there is a broad consensus on some of the critical genetic modifications. These include the elimination of porcine xenoantigens, and transgenic expression of human proteins to inhibit complement activation and coagulation dysregulation. Notably, a similar combination of genetic modifications, including *GGTA1*-KO, and transgenic expression of human CD46 and THBD, was used in the most successful preclinical trials in both heterotopic and orthotopic cardiac xenotransplantation, resulting in consistent long-term xenograft survival. We consider this combination as "minimum essential genetic modifications." However, similar success is yet to be demonstrated in clinical trials. More preclinical and clinical trials are needed to determine whether additional genetic modifications, beyond the minimum-essential ones, can synergize to enhance the efficacy of xenotransplantation. To minimize the potential side effects of extensive genetic modifications, as well as off-target effects from gene-editing tools, it is crucial to critically assess the necessity of each additional genetic modification. Some studies advocate for further genetic modifications to address cell-mediated rejection, eliminate PERVs, and regulate post-transplant organ growth. However, if an appropriate pig breed is chosen, certain genetic modifications can be avoided. For instance, Auckland Island pigs have a naturally small body stature and were selected to be free of PERV-C, eliminating the requirement for additional gene edits to regulate the organ size or remove PERVs.

Recently, the compassionate use of 10-GM porcine hearts and kidneys in humans achieved a maximum survival of 2 months.

Although this survival time is shorter compared to what was observed in preclinical trials, it marks a significant milestone in the field of xenotransplantation. Importantly, these source pigs had the aforementioned minimum essential genetic modifications as well as some additional ones. However, the published reports from these clinical studies do not provide details on the expression level of human transgenes in the xenografts. We speculate that the level of transgene expression might be more critical compared to the number of transgenes for achieving long-term survival and function of the xenograft. Nevertheless, these cases underscore the importance of continuous innovation in genetic modifications and immunosuppressive strategies for successful xenotransplantation.

The use of multimodal deep phenotyping techniques in xenotransplantation research has provided valuable insights into immune responses and potential improvements for the success of organ transplants from genetically modified pigs. By integrating various analytical approaches, such as histopathology, immunophenotyping, gene expression profiling, and spatial transcriptomics, researchers have been able to identify specific immune reactions and molecular changes associated with xenograft rejection or acceptance. Despite the limitations of small sample sizes and short study durations, the ongoing advancements in genetic engineering of donor pigs, coupled with the insights gained from preclinical and early clinical trials, are paving the way for xenotransplantation to become a viable and life-saving solution for patients in need of organ transplants.

AUTHOR CONTRIBUTIONS

AA and EW wrote the draft manuscript and created the figures. All other authors commented on the manuscript and approved the final version.

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CONFLICT OF INTEREST

Authors EK and EW are cofounders of the company XTransplant GmbH, Starnberg.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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The Evolution of Immunosuppressive Therapy in Pig-to-Nonhuman Primate Organ Transplantation

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An overview is provided of the evolution of strategies towards xenotransplantation during the past almost 40 years, focusing on advances in gene-editing of the organ-source pigs, pre-transplant treatment of the recipient, immunosuppressive protocols, and adjunctive therapy. Despite initial challenges, including hyperacute rejection resulting from natural (preformed) antibody binding and complement activation, significant progress has been made through gene editing of the organ-source pigs and refinement of immunosuppressive regimens. Major steps were the identification and deletion of expression of the three known glycan xenoantigens on pig vascular endothelial cells, the transgenic expression of human "protective" proteins, e.g., complement-regulatory, coagulation-regulatory, and anti-inflammatory proteins, and the administration of an immunosuppressive regimen based on blockade of the CD40/CD154 T cell co-stimulation pathway. Efforts to address systemic inflammation followed. The synergy between gene editing and judicious immunomodulation appears to largely prevent graft rejection and is associated with a relatively good safety profile. Though there remains an incidence of severe or persistent proteinuria (nephrotic syndrome) in a minority of cases. This progress offers renewed hope for patients in need of life-saving organ transplants.

Keywords: immunosuppression, xenotransplantation, transplantation immunology, swine, non-human primate

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INTRODUCTION

Despite the great progress that has been made in the gene-editing of pigs that are the sources of organs or cells for xenotransplantation in nonhuman primate (NHP) or human recipients, there remains a need for the administration of exogenous immunosuppressive therapy to the recipient of a pig xenograft [1]. Increased gene-editing of the organ-source pigs [2, 3] and the introduction of new agents that are more effective in suppressing the human immune response are both key factors that have allowed changes to be made to the immunosuppressive regimen to prevent rejection.

We here briefly review the changes in pre-transplant treatment, immunosuppressive protocols, and adjunctive therapy that have been made during the past almost 40 years of pig-to-NHP heart or kidney transplantation with some based on concomitant *in vitro* studies [4]. These observations are

Abbreviations: ATG, anti-thymocyte globulin; Gal, galactose-α1,3-galactose; GTKO, α1,3-galactosyltransferase gene-knockout; mAb, monoclonal antibody; MMF, mycophenolate mofetil; NHP, nonhuman primate; TKO, triple-knockout (i.e., with deletion of expression of the three known pig carbohydrate xenoantigens against which humans have natural antibodies.).

TABLE 1 | Glycan xenoantigens that have been deleted in gene-edited pigs.

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

TABLE 2 | Representative immunosuppressive regimen administered in the wild-type pig-to-NHP heterotopic heart Tx model [14].

Pre-transplant therapy
Ex vivo hemoperfusion of recipient's blood through a donor-specific pig kidney for 1 h (x2 kidneys)
Splenectomy (in some cases) 4–8 days before the transplant
Induction therapy
Cyclosporine by continuous IV infusion (15–32 mg/kg/day) until a therapeutic level of >400 ng/mL was achieved
Maintenance therapy
Cyclosporine by continuous IV infusion (15–32 mg/kg/day) to maintain therapeutic level of >400 ng/mL
Methylprednisolone 10 mg/kg/day IM (on day of transplant), with plan to taper the dose to 2 mg/kg/day
Outcome
Longest heart graft survival = 5 days

made largely through the experience of one group but that of several other groups has also been reviewed.

THE "CONVENTIONAL" IMMUNOSUPPRESSIVE THERAPY ERA

By the 1980s, it was known that natural (preformed) antibodies, when bound to antigens on a discordant animal organ graft, could activate complement, resulting in hyperacute rejection (defined as rejection occurring within 24 h) [5–10]. There was evidence that natural antibodies developed as a defense mechanism when the gastrointestinal tract of infants became colonized by microorganisms that expressed carbohydrate antigens, e.g., galactose-α1,3-galactose (Gal), that were also expressed on pig cells (**Table 1**) [11, 12].

Antibody Depletion

When xenotransplantation was first explored in wild-type (i.e., genetically-*unmodified*) pig-to-NHP models in the 1980s [13, 14], cyclosporine had become available, but tacrolimus was not yet accessible to most groups. Initial studies were therefore based on the regimens used in clinical allotransplantation, i.e., cyclosporine with added steroids with or without azathioprine or mycophenolate mofetil (MMF) (**Table 2**) [14]. The results were extremely disappointing and, with graft survival still measured in minutes, hours, or a few days, the administration of a cyclosporine-based regimen made little difference to the outcome. The innate immune response was clearly very strong and the effect of cyclosporine in suppressing the adaptive immune response was very modest (in contrast to its effect in allotransplantation).

As pre-transplant splenectomy was thought to be beneficial in allotransplantation across the ABO blood group barrier [15, 16], it was believed that it might also reduce the immune response to a pig graft, probably by removing a major source of B cells in the host as well as by decreasing the number of lymphocytes and their proliferative responses [17, 18]. With time, however, evidence for this was lacking and, possibly after the addition of rituximab to the protocol, it was eventually omitted from the regimen.

An effort was made to deplete the potential recipient of anti-pig antibodies either by 1) plasmapheresis [17], which extended graft survival to a maximum of 23 days, though usually for a shorter period of time, or by 2) preliminary perfusion of the recipient blood through a donor-specific second organ, e.g., the kidney, before donor-specific heart transplantation [13, 14], but graft survival remained very limited.

When Gal was clearly identified as the major target for human preformed anti-pig antibodies (**Table 1**) [19–23], techniques of antibody depletion were explored *in vitro* [24, 25] and refined to allow removal or "neutralization" of only anti-Gal antibodies, thus not depleting the NHP of antibodies that might be important in protecting from infectious complications. Again based on experience in overcoming the barrier of ABO-incompatibility, anti-pig antibody immunoadsorption was achieved by 1) perfusion of the recipient's blood through an immunoaffinity column of synthetic Gal oligosaccharides [20, 26–33] or 2) the continuous intravenous infusion of soluble synthetic Gal oligosaccharides [33–36].

In this latter approach, the infused synthetic Gal oligosaccharides would be bound by the circulating anti-Gal antibodies and therefore "neutralize" them by preventing them from binding to the pig graft. This approach had proven to be successful in preventing rejection of ABO-incompatible cardiac *allografts* in baboons [14]. Subsequently, modifications were made, e.g., synthetic Gal oligosaccharides were attached to bovine serum albumin which was then infused intravenously [37, 38]. All of these approaches prevented hyperacute rejection of the graft, but the return of antibody within days inevitably resulted in graft loss [39, 40].

In retrospect, the removal or neutralization of anti-Gal antibodies alone was probably misguided because, in addition to the early return of anti-Gal antibodies, there was already evidence of the presence of antibodies to non-Gal antigens [41–43] (identified as N-glycolylneuraminic acid [44] and Sda [45] [**Table 1**]). However, at the time, it was hoped that "accommodation" would develop [46] (i.e., when the return of antibody is no longer associated with rejection) as occurs in many patients receiving an ABO-incompatible allograft [47], but this proved not to be the case. The exact mechanism by which accommodation occurs remains uncertain. The additional

TABLE 3 | Representative immunosuppressive regimen administered in the hDAF (CD55) transgenic pig-to-NHP heterotopic heart and kidney Tx models [55].

Pre-transplant therapy
None
Induction
Cyclophosphamide 40 mg/kg on day −1 IV and 10 mg/kg on day 0 (the day of the transplantation) IV
Cyclosporine 35 mg/kg x2 daily orally from day −2
Methylprednisolone 1 mg/kg IV on day 0
Maintenance
Cyclosporine 35 mg/kg x2 daily orally to achieve a 12-hour trough level of 300–500 ng/mL
Prednisone 1 mg/kg orally on days 1 and 2 with subsequent tapering by 0.05 mg/kg/day to a baseline dose of 0.2 mg/kg/day from day 18
Outcome
Longest life-supporting kidney graft survival = 90 days
Longest non-life-supporting (heterotopic) heart graft survival = 62 days

differences in complement and coagulation factors between pig and human (see below) probably contributed to the difference in outcome between allograft and xenograft.

Protection From Complement Injury

Complement depletion or inhibition in the potential NHP recipient extended graft survival to a maximum of 25 days [48–52], but it was suspected that patients with no complement activity would be at risk for infectious complications and would not do well long-term and so this approach was not pursued (although prolonged complement inhibition has been adopted again by some groups recently [53]).

The introduction of the first gene-edited pigs by White and his colleagues at the British biotechnology company, Imutran, was a milestone in xenotransplantation research and enabled progress to be made [54]. These pigs expressed a single human complement-regulatory protein, CD55 (decay accelerating factor, DAF), and this alone extended kidney or heart graft survival in some immunosuppressed recipient NHPs for several weeks – in one case for up to 3 months [55]. However, the transplantation of hCD55 transgenic pig grafts proved successful only if intensive cyclosporine-based immunosuppressive regimens were employed (**Table 3**) [56]. For example, induction therapy with cyclophosphamide was found to be beneficial or even essential. It was later confirmed that the expression of a human complement-regulatory protein helps protect the graft from systemic complement activation in the host [57].

Depletion of complement for a prolonged period of time was, and still is, considered to put the patient at increased risk of infectious complications. Once the transgenic expression of human complement-regulatory proteins could be induced in the organ-source pig, systemic complement inhibition was avoided by most groups. However, transient systemic complement inhibition at the time of pig organ transplantation, when there is complement activation and inflammation, may be beneficial [58]. When introduced by Langin et al [59], the administration of a C1-esterase inhibitor on just 2 days appears to be safe and beneficial, even though it has not been conclusively demonstrated to be essential. Long-term complement inhibition, e.g., with a C5 inhibitor, has been incorporated into the immunosuppressive regimen by some groups [53], but its necessity remains controversial.

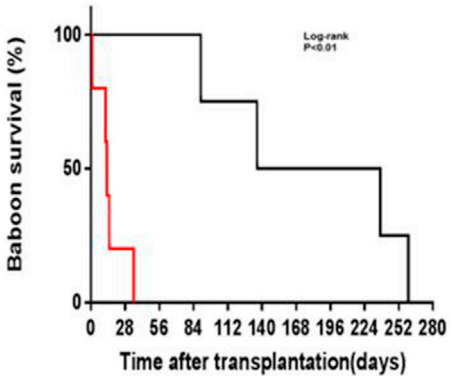


FIGURE 1 | GTKO pig kidney survival in baboons receiving US FDA-approved immunosuppressive agents (Group A, in red) was much shorter than in those receiving an anti-CD40mAb-based regimen (Group B, in black) outlined in **Table 6** (Reproduced with permission from Yamamoto T, et al. [81]).

THE INTRODUCTION OF CD40/CD154 T CELL CO-STIMULATION PATHWAY BLOCKADE

Based on encouraging studies in models of allotransplantation, in 2,000 Buhler et al. carried out pig hematopoietic cell transplantation in an attempt to induce chimerism as a basis for achieving immunological tolerance to a pig organ in an immunosuppressed NHP [60, 61]. When immunosuppressive therapy was based on cyclosporine, an elicited antibody response to the pig cells was clearly detected within the first 14 post-transplant days. In contrast, treatment with an anti-CD154 monoclonal antibody (mAb) prevented this antibody response [60] (Blockade of the B7/CD28 pathway did not prove equally successful) [62–65]. This proved a major step forward. Since then, almost all groups have employed an anti-CD154 or anti-CD40mAb as the basis of their immunosuppressive regimen [53, 66–77].

Yamamoto et al. subsequently demonstrated prolonged survival of kidney grafts from α1,3-galactosyltransferase gene-knockout (GTKO) pigs (in which the most important xenoantigen, galactose-α1,3-galactose [Gal] against which humans and NHPs have natural antibodies had been deleted [78–80] in NHPs receiving a CD40/CD154 co-stimulation-based inhibitory regimen compared to NHPs receiving a conventional (tacrolimus-based) immunosuppressive regimen (**Figure 1**) [81]. The administration of an anti-CD154mAb has been associated with better results when compared with an anti-CD40mAb [74]. In summary, by modulating the immune response between T cells and antigen-presenting cells, inhibiting co-stimulatory pathways improves long-term post-transplant outcomes.

When attempting to induce tolerance to an allotransplant, at that time the potential recipient was pre-treated with whole body and thymic irradiation. However, in the pig-to-NHP model,

TABLE 4 | Representative immunosuppressive regimen administered in the GTKO pig-to-NHP heterotopic heart Tx model [82].

Pre-transplant therapy
Thymic irradiation 700 cGy (day −1)
Induction
Anti-thymocyte globulin (horse ATG [ATGAM])* 50 mg/kg IV on days −3, −2, and −1 (3 doses)
LoCD2b** 1–4 mg/kg IV on days 1–7
Cobra venom factor (CVF) 6 mg/day IV for 4–15 days in some cases
Maintenance
Anti-CD154mAb (AB1793, Novartis) 25 mg/kg IV
Mycophenolate mofetil (MMF) 25–110 mg/kg/day by continuous IV infusion from day −2
Methylprednisolone 4 mg/kg IM daily reducing to 0.5 mg/kg/day
Heparin 5–60 U/kg/h IV from day 0
Aspirin 40 mg on alternate days from day 4 (in some cases)
Outcome
Survival from 2 to 6 months (median 78 days)

To deplete T cells, the necessary dose of horse ATG [ATGAM] is significantly higher than of rabbit ATG (Tables 5**, **6**).*

***LoCD2b depletes cells expressing CD2 that include T and NK cells [157].*

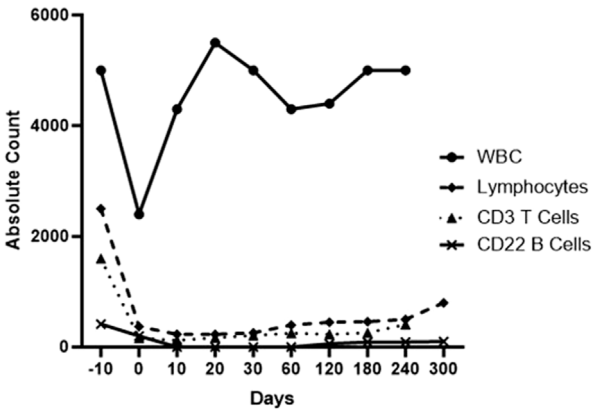


FIGURE 2 | Total white blood cell, lymphocyte, T cell, and B cell counts in the blood of a baboon with a life-supporting pig kidney that received the immunosuppressive regimen outlined in **Table 6**.

survival of the graft rather than tolerance induction was the major aim, and so whole body irradiation was deemed unnecessary, although thymic irradiation was still carried out for a period of time (**Table 4**).

Nevertheless, induction therapy was still considered essential. Initially, this consisted of large doses of anti-thymocyte globulin and other agents that depleted T cells (**Table 4**). With the transplantation of organs from pigs expressing fewer xenoantigens and a greater number of human protective transgenes it was determined that the dose of ATG could be reduced. The effect of the ATG can be determined simply by following the total lymphocyte count.

However, additional B cell depletion was considered beneficial and so the administration of an anti-CD20mAb (in the form of Rituximab) was included, initially by McGregor et al [83]. Whether this is essential remains uncertain but a significant reduction in B cells in the blood for several weeks (**Figure 2**) may possibly result in a subsequent reduction in plasma cells, though this has not been proven.

The initial anti-CD154mAbs were tested *in vitro* and *in vivo* [75, 84–86]. When transplants were still being carried out with organs from wild-type pigs, the results remained disappointing because the innate response remained strong. When GTKO pigs became available, however, the results improved markedly. The first series that combined the transplantation of heterotopically-placed hearts (i.e., *not* life-supporting) from GTKO pigs into baboons with immunosuppression based on CD40/CD154 co-stimulation pathway blockade demonstrated greatly improved graft survival, extending to a maximum of 6 months (**Table 4**) [82, 87]. However, the recipient baboons were selected on the basis of their low anti-pig antibody levels.

Using an identical immunosuppressive regimen, life-supporting GTKO kidney grafts survived approximately only half as long [88], possibly because 1) kidneys may be more immunogenic than hearts, or 2) the kidneys were life-supporting whereas the hearts were not. When GTKO pig kidneys were transplanted into NHPs immunosuppressed with a tacrolimus-based regimen, the results were markedly inferior [89].

The transplantation of GTKO hearts that expressed a different human complement-regulatory protein, CD46, with the same or very similar immunosuppressive regimen reduced early graft failure but did not extend maximum graft survival [90, 91].

The withdrawal of the original anti-CD154mAbs because of their thrombogenic effect [92–94] necessitated the use of anti-CD40mAbs, first introduced into xenotransplantation by Mohiuddin et al [68–70]. However, increasing data indicate that anti-CD154 agents are superior to anti-CD40 agents in preventing both the adaptive immune response and some aspects of the innate response [75, 95]. Once Fc-modified anti-CD154 agents (that do *not* result in platelet activation) were introduced [74, 75, 96–98], these soon became the treatment of choice [74, 76, 77].

One important observation made in regard to anti-CD154mAb therapy was that in infant baboons in which natural antibodies had not yet developed, treatment with an anti-CD154mAb prevented the development of natural anti-Gal and anti-AB antibodies, suggesting that natural antibodies may be, at least in part, T cell-dependent [99]. This has considerable relevance to the treatment of neonates with complex, life-threatening congenital heart disease, e.g., single ventricle physiology, by pig heart xenotransplantation [100, 101]. For example, by inhibiting both natural and elicited antibody production, treatment with an anti-CD154mAb during the first week of life (at the time of pig heart transplantation) might possibly facilitate the development of immunological tolerance to the graft. Once the graft has been established, it may be possible to discontinue all immunosuppressive therapy.

Despite the suppressive effect of agents that block the CD40/CD154 co-stimulation pathway, the transplantation of kidneys or hearts from GTKO pigs was not consistently successful, with some grafts failing from antibody-mediated rejection [66, 82, 88, 102–105], coagulation dysfunction, or graft vasculopathy (chronic rejection) (**Figure 3**) [106].

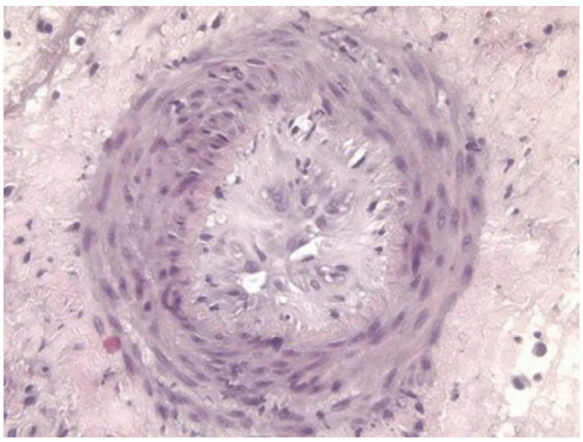


FIGURE 3 | Histopathological features of graft vasculopathy (chronic rejection) In a GTKO pig heart transplanted heterotopically (in the abdomen) 3 months previously in a baboon that received the immunosuppressive regimen outlined in **Table 4**.

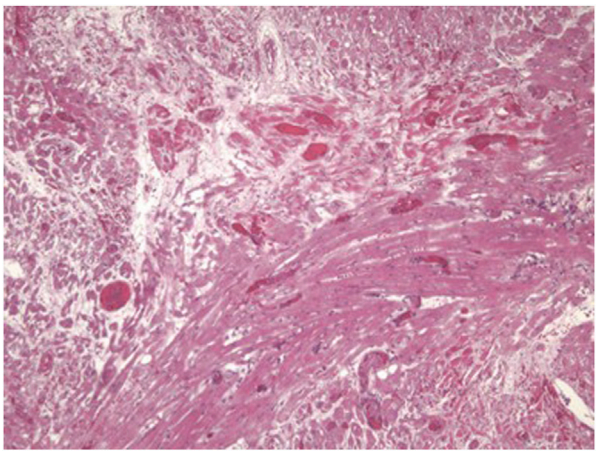


FIGURE 4 | Histopathological features of a pig cardiac graft demonstrating multiple vascular thromboses with surrounding ischemic changes (fibrosis) in a baboon that received the immunosuppressive regimen outlined in **Table 4**.

Protection From Coagulation Dysfunction

Predictions of significant differences in the coagulation-anticoagulation systems between pig and human had been discussed for some time [107, 108], but evidence in the important pig-to-NHP model was first reported in the late 1990s [84, 109–111] (**Figure 4**). Although the presence of porcine cytomegalovirus (CMV) in the graft was identified as playing a role in coagulation dysfunction [112], later confirmed by Yamada [113], this problem stimulated the need to introduce human coagulation-regulatory genes into the pig. Thrombomodulin, endothelial cell protein C receptor (EPCR), and/or tissue factor pathway inhibitor (TFPI) were expressed in the pig.

When the problems relating to coagulation dysfunction between pig and human were confirmed, the introduction of GTKO. hCD46 pigs that additionally expressed human thrombomodulin reduced the incidence of thrombotic microangiopathy in the pig graft and of consumptive coagulopathy in the recipient NHPs (**Table 5**; **Figure 5**) [70]. This prolonged life-supporting kidney graft survival to 7–8 months, with termination of the experiments from infectious complications rather than from rejection [70].

Protection From Systemic Inflammation

Xenotransplantation was found to be accompanied by a systemic inflammatory response that could be detrimental to the survival of the graft by augmenting the immune response and/or coagulation dysfunction [62, 114–118]. Corticosteroids appeared to have no effect in suppressing this response.

However, in the period 2015-2020, the beneficial effects of interleukin-6 receptor (IL-6R) blockade using tocilizumab (which blocks IL-6 binding to the receptors on NHP cells but *not* on pig cells) were investigated [119–121]. Although the initial results were encouraging, the accompanying rise in IL-6 in the blood engendered some caution in the use of tocilizumab [120, 121]. With additional experience, we have tentatively concluded that this agent has a positive effect on graft survival [77]. It may be particularly beneficial when orthotopic pig heart transplantation is carried out because it may protect the *recipient's t*issues, e.g., the lungs, from inflammatory injury associated in part to the need for cardiopulmonary bypass [118]. However, the combination of two inhibitors of IL-6 proved fatal from profound thrombocytopenia [122].

There is some evidence that the introduction of a human "anti-inflammatory" transgene into the pig, e.g., hemeoxygenase-1, A20, has a protective effect on the graft [3, 121, 123].

Low-dose corticosteroids have been used in almost all regimens (probably because they are included in the regimens of most patients with organ allografts) but there is little evidence that they are essential when co-stimulation blockade is employed, particularly if tocilizumab is being administered. As long ago as 2005, Yamada carried out one GTKO pig kidney transplant in a baboon without maintenance steroids and found no significant detrimental effect on graft survival [88]. Our suspicion is that steroids add little to the efficacy of the regimen, particularly if it includes tocilizumab.

Although not fully recognized in the early days of xenotransplantation research, inhibition of complement activity also has beneficial effects on coagulation dysfunction and the inflammatory response (see below) [58].

ADDITIONAL IMMUNOSUPPRESSIVE THERAPY AND ADJUNCTIVE THERAPY

Although blockade of the CD40/CD154 co-stimulation pathway has formed the basis of all effective regimens for the past two decades [60], its dosage is important. For example, dosing of the Tonix-1500 anti-CD154mAb at 20 mg/kg weekly, although effective in preventing rejection of allografts, was not entirely successful in regard to grafts from triple-knockout (TKO) pigs, in

TABLE 5 | Representative immunosuppressive regimen used in the GTKO/CD46/hTBM pig-to-NHP life-supporting kidney Tx model [70].

Pretransplant therapy
None
Induction
Anti-thymocyte globulin (ATG) 5 mg/kg IV on day –2 (i.e., 2 days before kidney transplantation)
Anti-CD20 mAb (Rituximab) 10 mg/kg IV on day –1
C1 esterase inhibitor 17.5 units/kg IV on days 0 and 2
Maintenance
Anti-CD40 mAb 20 mg/kg IV on days 0, 2, 7, 10, 14, and weekly
Rapamycin x2 daily IM to maintain a 12-hour trough level of 6–12 ng/mL
Methylprednisolone 10 mg/kg IV tapering the dose over the first week to 0.25 mg/kg IM daily
Anti-TNF mAb (etanercept) (in some cases)
Tocilizumab (IL-6R blockade) 8 mg/kg IV monthly for 6 months
Outcome
Two grafts functioned for >7 and >8 months, respectively, with the experiments being terminated for infectious complications
(When human thrombomodulin (hTBM) was *not* expressed in the kidney, a consumptive coagulopathy developed within 12 days, necessitating euthanasia)

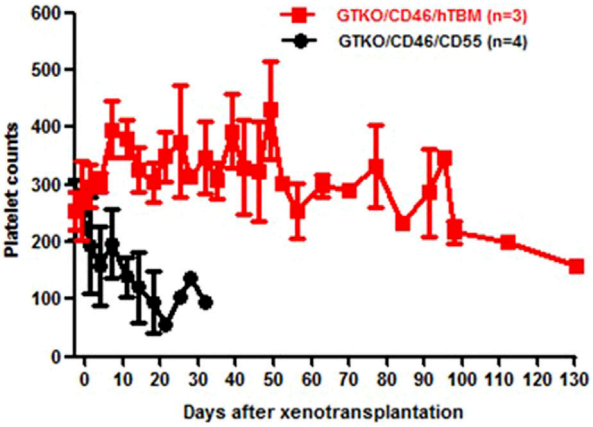


FIGURE 5 | Platelet counts in baboons with hearts from GTKO.CD45.TBM (red) or GTKO.CD46.CD55 (black) pigs. The baboons received the immunosuppressive regimen outlined in **Table 5**.

which expression of all three of the known pig xenoantigens against which humans have natural antibodies has been deleted (**Table 1**) [74]. A higher dosage, however, appears to be consistently successful without significant infectious complications [77] and Kinoshital et al. (unpublished data).

It must be remembered that *all* Old World NHPs have natural antibodies to TKO pig cells, thus increasing the hurdle that has to be overcome, i.e., the hurdle of "sensitization" (that will *not* be the case in many human patients receiving a pig xenograft) [124–127]. The results of TKO pig organ transplantation in NHPs are inferior to those of GTKO pig organ transplantation [71, 128], but remarkably CD40/CD154 co-stimulation pathway blockade appears to overcome this hurdle if recipient NHPs are selected with low anti-pig antibody levels (**Table 6**) [72, 77, 129].

Whether other agents, if any, need to be combined with co-stimulation blockade remains uncertain. Pierson and Kawai and their respective colleagues have clearly demonstrated that in *allo*transplantation no other agents (either for induction or maintenance) may be necessary because anti-CD154mAb alone (in the form of Tonix-1500) prevents rejection almost consistently for at least 6 months (at which time the studies have been electively concluded) [97, 98]. Rejection develops only some weeks or months after cessation of treatment. There is some evidence, however, that the addition of low-dose rapamycin or tacrolimus to the regimen ensures an absence of rejection.

However, these agents alone are not so effective in xenotransplantation. For example, Tonix-1500 alone (with *no* induction therapy or additional maintenance therapy) was associated with antibody-mediated rejection of a kidney graft from a pig with 10 gene-edits on post-transplant day 4 [77]. This indicated to us that some form of induction therapy and additional maintenance therapy is required (**Table 6**).

When both T and B cells are depleted by this induction therapy (**Table 6**), the anti-CD40 or anti-CD154mAb maintenance therapy appears to maintain low lymphocyte counts throughout the first 6 months of the post-transplant period (**Figure 2**), which we suspect contributes to preventing an immune response to the graft [70, 77, 130].

To augment the effect of anti-CD40 or anti-CD154mAbs, we have selected rapamycin, in part because it can be administered intramuscularly, which is an advantage when managing NHPs that do not consistently take oral medications [131]. Mammalian target of rapamycin (mTOR) inhibitors have several properties that may be especially beneficial in xenotransplantation, e.g., suppression of T cell proliferation, increases in the number of T regulatory cells, inhibition of pig graft growth, and anti-inflammatory, anti-viral, and anti-cancer effects [132].

However, rapamycin is not tolerated by some patients (largely from gastrointestinal disturbances or oral ulcers) and so other pharmacologic immunosuppressive agents have been incorporated in several regimens. MMF has perhaps been the most commonly used agent [74, 76] but its value, as with rapamycin and tacrolimus, has *not* been proven. No group has yet had the courage to maintain immunosuppression with co-stimulation blockade alone. However, preliminary evidence that this may be possible was reported in one baboon when all immunosuppressive therapy, except anti-CD40mAb, was discontinued 2 months after pig kidney transplantation. During follow-up for a further 2 months, no clinical or histopathological features of rejection were observed [133]. Nevertheless, we have seen antibody-mediated rejection on occasions when the rapamycin level fell to subtherapeutic levels.

One important observation made recently is that, when proteinuria is present (which may be an early sign of antibody-mediated rejection), therapeutic mAbs may be lost in the urine, thus exposing the xenograft to rejection [77]. Furthermore, there is some evidence that an infectious microorganism in a xenograft (e.g., pyelonephritis) may induce an immune response, resulting in rapid rejection as has been well-documented in ABO-incompatible kidney allotransplantation [134, 135].

TABLE 6 | Representative immunosuppressive regimen used in the TKO (+added transgenes) pig-to-NHP life-supporting kidney Tx model [77].

Pretransplant therapy
None
Induction
Anti-thymocyte globulin (ATG), 5 mg/kg IV on day −2 (i.e., 2 days before kidney transplantation [day 0])
Anti-CD20mAb (Rituximab) 10 mg/kg IV on day −1
C1 esterase inhibitor 17.5 units/kg IV on days 0 and 2
Maintenance
Anti-CD154mAb (Tonix-1500), 30 mg/kg IV on days 0, 2, 7, 10, 14, and weekly
Rapamycin daily IM to maintain a 24-hour trough level of 8–12 ng/mL
Methylprednisolone 10 mg/kg IV tapering the dose over the first week to 0.25 mg/kg IM daily
Tocilizumab (IL-6R blockade) 8 mg/kg IV monthly for 6 months
Outcome
Maximum ongoing graft function is now >12 months

COMMENT

Throughout the early years covered by this brief report, researchers searched for other agents that might suppress the production of anti-pig antibodies. These agents included 1) various known immunosuppressive agents [30], 2) drugs used in other conditions but thought to have immunosuppressive properties [136–142], 3) new monoclonal antibodies directed towards depletion of plasma cells [143], 4) anti-idiotypic antibodies [144, 145], and 5) agents that influence expansion of T regulatory cells, but the majority proved unworthy or unnecessary of inclusion in the immunosuppressive regimen. An important observation was that prolonged treatment with bortezomib (a proteosome inhibitor) to patients who were highly sensitized to HLA had only a minimal effect in reducing anti-pig antibody levels [142].

Two agents that might well be valuable when xenotransplantation is introduced into the clinic are 1) atorvastatin [140, 141], whose anti-inflammatory effect could be of value. (As only tablets were available to us, we found it difficult to administer it successfully to NHPs.) and 2) alemtuzumab - but administering it to NHPs has several major limitations [146].

With the aim of protecting their cells from the adaptive immune response, gene-editing of the organ-source pigs has been explored. This included producing pigs that secreted CTLA4-Ig [147, 148] and pigs in which Major Histocompatibility Complex (MHC) Class II expression had been downregulated [149].

The level of CTLA4-Ig in the blood in pigs expressing CTLA4-Ig was approximately 10-fold higher than the therapeutic level in humans being treated with the agent [147]. Although this demonstrated the success of the gene-editing, the pigs were rendered immunocompromised and developed infections at a relatively young age for which they required euthanasia. This clearly precluded them from acting as sources of organs and from breeding. Furthermore, we concluded that maintaining the correct level of immunosuppression after organ transplantation in NHPs would also be difficult. However, others have successfully expressed CTLA4-Ig in the pancreatic islets [150, 151] although this limited expression may not be sufficient to protect against T cell-mediated rejection. MHC-Class II-knockdown was successful in reducing the T cell response and is worthy of further exploration [65, 149].

The currently available gene-edited pigs that are TKO and also express multiple human proteins [3, 76] would appear to be sufficient for clinical trials to be undertaken today. Future gene-editing may include the introduction of HLA-E and G [151], PD-L1 [152, 153], and MHC Class 1 knockout [154] or MHC Class II modification [155, 156].

Although gene-editing of pigs has been the major factor that has enabled progress to be made in pig organ xenotransplantation in NHPs, the introduction of immunosuppressive agents directed towards blockade of the CD40/CD154 co-stimulation pathway must not be underestimated. Successful clinical pig organ transplantation will require a combination of judicious gene-editing and the administration of an effective, but not excessive, immunosuppressive regimen. We suggest that selecting recipients with low levels of anti-pig IgM and preferably no IgG will contribute to success.

AUTHOR CONTRIBUTIONS

The topic was suggested by DC and SS. All authors contributed to gathering relevant evidence and drafting the manuscript.

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CONFLICT OF INTEREST

DC is a consultant to eGenesis Bio of Cambridge, MA, but the opinions expressed in this article are those of the authors, and do not necessarily reflect those of eGenesis.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GENERATIVE AI STATEMENT

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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REVIEW
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Liver Xenotransplantation: A Path to Clinical Reality

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Liver xenotransplantation has emerged as a potential solution to the shortage of deceased human donor organs and is now becoming a reality due to recent developments in genetic engineering and immunosuppressive therapy. Early efforts using non-human primates and genetically modified pigs faced significant challenges such as thrombocytopenia and graft rejection. Understanding the mechanism behind those challenges and using novel genetically engineered pigs enabled researchers to overcome some of the hurdles, but more research is needed. However, new advances might allow pig liver xenotransplantation to potentially serve as a bridge to liver allotransplantation or allow native liver regeneration in the near future.

Keywords: coagulation dysregulation, liver, pig, platelets, xenotransplantation

INTRODUCTION

Liver diseases are a significant cause of morbidity and mortality. Although advances in antiviral therapy have decreased morbidity and mortality from viral hepatitis, the number of patients with non-alcoholic fatty liver disease is increasing significantly due to the rising obesity epidemic. According to National Health and Nutrition Examination Surveys, its prevalence has risen from 20% between 1988 and 1994 to 32% between 2009 and 2012 [1]. Moreover, the prevalence of alcoholic liver disease is rising due to increased alcohol consumption in the world [2]. As a result, the incidence of end-stage liver disease and related cirrhosis has substantially increased which has accentuated the donor liver shortage.

Liver transplantation provides a potential cure for patients with end-stage liver disease. However, due to the limited availability of organ donors, a significant number of patients die on the waiting list from complications related to cirrhosis [3]. In addition to the scarcity of organ donors with a transplantable liver, the allocation and transportation of liver allografts pose another significant burden. According to the United Network for Organ Sharing (UNOS) of the United States, the median waiting time for the most urgent liver transplant listing, which is considered a national emergency (Liver Status 1A) in cases of acute or fulminant liver failure, is 6 days. In the case of acute liver failure, only 55% of patients survive without liver transplantation [4]. Between 2011 and 2014, the median time for a non-urgent listing for liver transplantation for patients aged between 18 and 34 years was 1,048 days, for those 35–49 years was 1,080 days, and for those 50–64 years was 1,136 days [5]. After a recent change in liver allocation, the median waiting times are yet to be announced by the UNOS.

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Abbreviations: CMV, cytomegalovirus; GTKO, α1,3-galactosyltransferase gen-knockout; hDAF, human decay-accelerating factor; mAb, monoclonal antibody; NHP, non-human primate.

TABLE 1 | World experience with clinical liver xenotransplantation (1966–2024).

Surgeon/ Year	Recipient age/ sex (M = male, F = female)	Donor animal	Underlying pathology	Surgical technique	Preventive measures and immunosuppressive therapy	Outcome
Starzl/ 1966 [12]	28 months/M	Chimpanzee	Intrahepatic biliary atresia	OLT	Azathioprine, prednisone, antithymocyte globulin	Evidence of function was seen in hepatic xenograft, the patient was lost at POD 9 due to severe infections from excessive immunosuppression
Starzl/ 1969 [13]	7 months/M	Chimpanzee	Extrahepatic biliary atresia	OLT	Liver transplantation with both donor kidneys, receiving systematic arterial supply and draining to portal system	Death at 26th hour from brain edema, child never woke up from surgery. There was evidence of liver xenograft function postoperatively and no evidence of hepatocellular damage on autopsy
Bertoye/ 1969 [14]	22 years/F	Baboon	NA	HLT	NA	Survived for more than 4 months
Bertoye/ 1969 [14]	7 months/M	Baboon	NA	HLT	NA	Died at 39th hour after surgery
Marion/ 1970 [15]	NA/NA	Baboon	NA	HLT	NA	Survived less than 24 h
Leger/ 1970 [16]	23 years/F	Baboon	NA	HLT	NA	Survived for 72 h
Pouyet/ 1971 [17]	28 years/F	Baboon	NA	HLT	NA	Survived for less than 2 days
Pouyet/ 1971 [16]	34 years/F	Baboon	NA	HLT	NA	Survived for less than 2 days
Motin/ 1971 [16]	NA/NA	Baboon	NA	HLT	NA	Survived for 3 days
Starzl/ 1974 [18]	23 months/NA	Chimpanzee	Persistent acute rejection following liver allotransplantation (for biliary atresia)	OLT	NA	Survived 14 days. No evidence of hyperacute rejection. Centrilobular cholestasis
Starzl/ 1992 [18]	35 years/NA	Baboon	Decompensated cirrhosis from hepatitis B infection	OLT	Tacrolimus, cyclophosphamide	Death from subarachnoid hemorrhage caused by invasive aspergillus infection at day 70
Starzl/ 1993 [18]	62 years/M	Baboon	Decompensated cirrhosis from hepatitis B infection	OLT	Tacrolimus, cyclophosphamide	Anastomotic leak and bile peritonitis at day 26
Makowka/ 1993 [19]	26 years/F	Wild-type pig	Fulminant hepatic failure from autoimmune hepatitis	HLT	Plasmapheresis	Rapid rise in xenoantibody levels and liver failure after 3 h
Beicheng/ 2024 [20]	71 years/M	10-gene pig	Large liver cancer	HLT	NA	After 2 weeks, patient had good liver function. No further information

The table was modified from Taniguchi and Cooper [17].
HLT, heterotopic liver transplantation; NA, not available; OLT, orthotopic liver transplantation; POD, post-operative day.

Liver transplantation is now offered as a new treatment option for patients with unresectable liver metastasis from colorectal cancer, which causes an increase in demand while supply stays almost the same [6]. In fact, there have been >50,000 patients who either died or were removed from the waiting list for being 'too sick to transplant' within the last 20 years [5].

Xenotransplantation, which is the transplantation of organs between different species, offers a potential solution to the scarcity of organs, providing a potentially unlimited supply of organs [7]. However, xenotransplantation brings challenges because of immunological barriers, interspecies incompatibility, coagulation dysregulation, and ethical problems. Advances in genetic engineering and improved immunosuppressive treatments have allowed us to overcome many challenges [8]. In this review, we summarize recent progress in clinical xenotransplantation, and in overcoming the immunological barriers and coagulation dysregulation (with a special focus on thrombocytopenia and coagulopathy), as well as a brief discussion of the ethical considerations. We also provide a summary of previously performed clinical liver xenotransplantation as a basis for creating a path to clinical liver xenotransplantation in the near future.

RECENT PROGRESS IN CLINICAL ORGAN XENOTRANSPLANTATION

Recently, there have been multiple significant developments in clinical xenotransplantation. The first (in 2022) was a pig-to-human cardiac xenotransplant using a 10-gene genetically-engineered pig heart and a novel co-stimulation blockade agent, anti-CD40 monoclonal antibody (mAb), carried out at

the University of Maryland at Baltimore [9]. The recipient of the pig heart lived for 61 days. The first clinical pig kidney transplant was carried out at the Massachusetts General Hospital in 2024. A kidney from a similar gene-edited pig was transplanted into a 62-year-old male patient with end-stage renal disease who lived for 52 days before dying of a cardiac complication ([10]; Kawai T et al, manuscript submitted).

Initial Experience in Clinical Liver Xenotransplantation

Developments in liver xenotransplantation have been slower even though, given its emergency and severity nature, acute liver failure requires urgent attention. One key reason is that liver xenotransplantation is more challenging than kidney and heart xenotransplantation due to major problems with thrombocytopenia and coagulopathy in addition to the immunological barriers to xenotransplantation [11].

Initial efforts to transplant livers from non-human primates (NHPs) into human recipients began in the 1960s (**Table 1**). Allotransplant pioneer, Thomas Starzl, and his colleagues performed three chimpanzee liver transplants in humans between 1966 and 1974, with the grafts functioning from only 1–14 days [21]. However, in 1993, he achieved a significantly longer survival when a 35-year-old man with chronic hepatitis B and HIV infection received a liver transplant from a baboon (**Table 1**) [22]. The surgical procedure followed conventional techniques, with immunosuppression using a regimen of tacrolimus, prednisone, prostaglandin, and a non-myelotoxic dose of cyclophosphamide. Despite the challenges of transplantation between species, the patient showed little evidence of hepatic rejection, with biochemical monitoring and histopathological examination indicating effective liver function for 70 days post-transplantation. The transplanted baboon liver produced clotting factors and other proteins without adverse effects. Unfortunately, the patient died of a cerebral hemorrhage due to an invasive aspergillus infection, but there was underlying widespread biliary sludge formation in the biliary tree [22].

One of the most critical steps in xenotransplantation research was realizing that NHPs are not ideal sources of organs for clinical xenotransplantation [23]. Pigs are better candidates than NHPs for several reasons, including their similarity in size, anatomy, and physiology, though, unless genetically-engineered, they are at a distinct disadvantage immunologically. Most recently in 2024, a genetically engineered (10-gene-edited) pig liver was transplanted into a 71-year-old living male patient who suffered from a large liver tumor that could be removed but would leave him with an inadequate remnant of native liver that would not sustain life [20]. Therefore, a 514-gram whole pig liver was transplanted in an auxiliary fashion to augment the function of the remainder of the native liver tissue while it regenerated and grew. Although the surgeons reported that the patient was doing well with good liver function for 2 weeks after the clinical pig liver xenotransplantation, no further formal scientific data have yet been reported [20].

IMMUNOLOGICAL BARRIERS

After identifying a suitable species as a source of organs for clinical xenotransplantation (the pig), the next major challenge was overcoming the immunological barriers. The innate and adaptive immunological barriers to xenotransplantation have been discussed previously by others [24]. Briefly, the innate immunological barriers include hyperacute rejection, associated with the presence of natural anti-pig (mainly directed to Gal antigens – see below) antibodies, complement activation, neutrophil infiltration, NK cell-mediated cytotoxicity, and macrophage activation [24]. The adaptive response includes the T cell response, including direct recognition of MHC class I on xenograft cells by $CD8^+$ and $CD4^+$ T cell activation, leading to proinflammatory cytokine release, and B cell activation [25]. Disruption of endothelial cells and dysregulation of the coagulation cascade caused by immunological activation are among the major challenges of pig liver xenotransplantation [24].

The initial focus of xenotransplant research was genetically modifying the pig to overcome the complement response, especially with the expression of humanized complement-regulatory proteins (CD55 or decay-accelerating factor) [11, 26, 27]. However, the identification of galactose-α-1,3-galactose (Gal), the major antigen present in pigs (but absent in humans), was among the most significant steps in xenotransplantation research [28–30]. Galili et al. and Good et al. independently demonstrated that humans have naturally existing anti-Gal antibodies that bind to Gal antigens on pig vascular endothelial cells, which was crucial for understanding the mechanism of hyperacute rejection [28–30]. When this was known, it was suggested that expression of Gal in pigs should be deleted by gene editing [31], although this was not possible at that time. It was not until a decade later that this significant problem was overcome through genetic engineering with the generation of α1,3-galactosyltransferase gene-knockout (GTKO) pigs [32–34].

There was a marked increase in the survival of NHPs that received a heart or kidney from a GTKO pig [35, 36]. GTKO pigs prevented hyperacute rejection and extended pig heterotopic (non-life-supporting) heart survival in baboons for up to 6 months [35]. With induction therapy, including at that time thymectomy, splenectomy, and T cell depletion, and maintenance therapy with an anti-CD154 monoclonal antibody (mAb), and mycophenolate mofetil with or without low-dose steroids, life-supporting kidney xenografts survived for up to 83 days with normal creatinine levels [36]. Similar success was anticipated from liver xenotransplantation, but this was not the case.

Pig-to-NHP Liver Xenotransplantation

Initial studies that used genetic modification technology in liver xenotransplantation were reported by Ramirez et al. [27]. The livers came from pigs expressing human CD55 to protect from complement activation. Two recipient NHPs survived for 4 and 8 days, but thrombocytopenia and coagulopathy were significant challenges [27]. Liver xenografts from GTKO pigs or GTKO pigs transgenic for human CD46 were implanted in baboons and survived for up to 7 days [37]. Even though the baboons died from bleeding associated with thrombocytopenia, liver function,

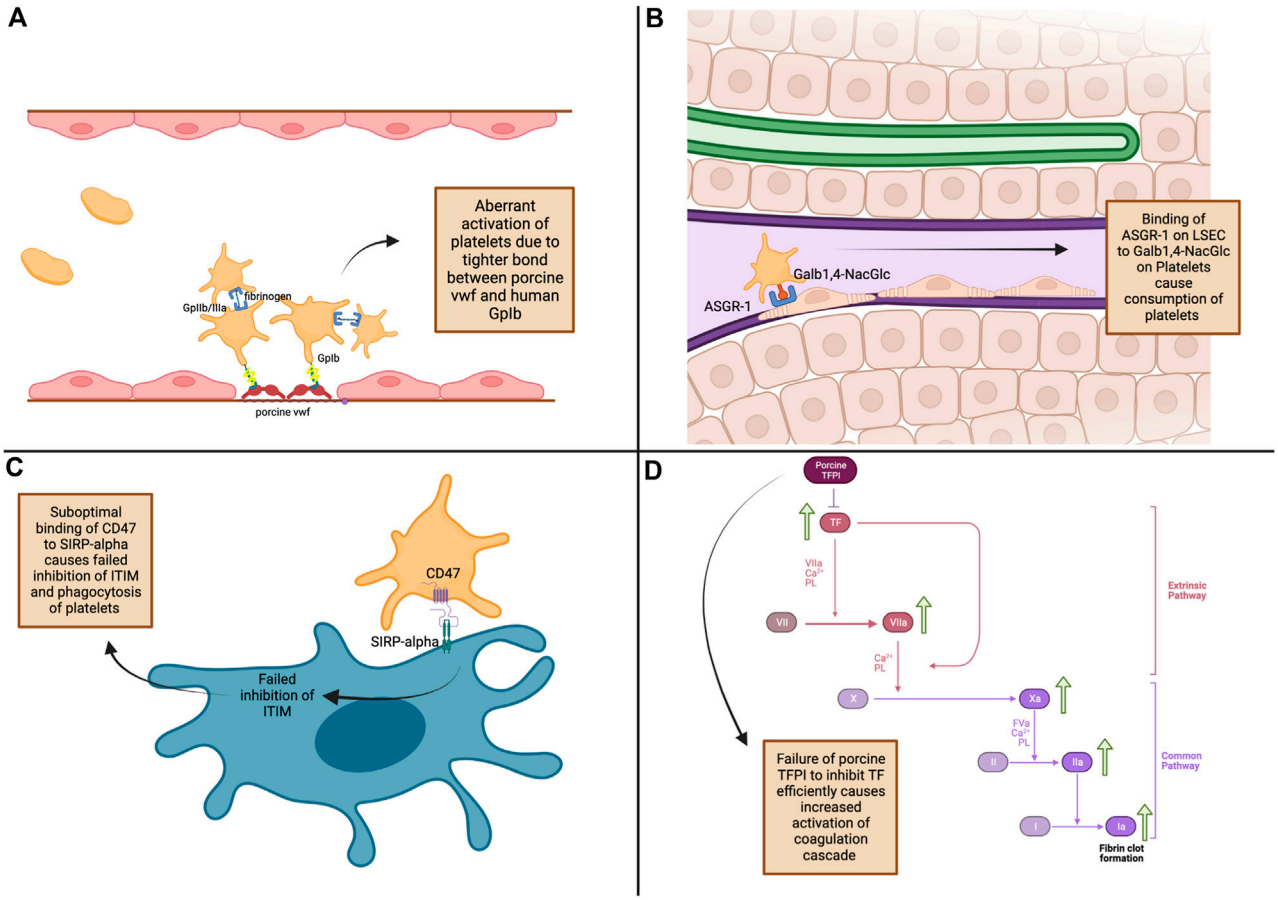


FIGURE 1 | Mechanisms of thrombocytopenia and coagulation dysregulation in pig-to-non-human primate liver xenotransplantation. **(A)** - Platelet Activation: Tight binding of porcine von Willebrand factor (vWF) to human GpIb receptors leads to increased platelet activation [11]. **(B)** - Platelet Consumption: ASGR1 on porcine liver sinusoidal endothelial cells binds to Galβ1,4-NacGlc on human platelets, causing platelet consumption [39]. **(C)** - Failed Phagocytosis Inhibition: Inadequate binding of porcine SIRP-α to human CD47 leads to phagocytosis of platelets [19]. **(D)** - Coagulation Cascade Activation: Porcine TFPI inefficiently inhibits tissue factor, leading to increased coagulation and fibrin clot formation [41]. ASGR1, asialoglycoprotein receptor-1; CD47, Cluster of Differentiation 47; Galb1,4-NacGlc, galactose β1-4 N-acetyl glucose; GpIIb/IIIa, glycoprotein IIb/IIIa; GpIb, glycoprotein Ib; ITIM, iImmunoreceptor tyrosine inhibitory motifs; LSEC, liver sinusoidal endothelial cells; SIRP-α, signal regulatory protein α; TF, tissue factor; TFPI, tissue factor pathway inhibitor; vWF, von Willebrand factor.

including coagulation and synthetic functions, were within an acceptable range [37]. Using GTKO pigs and demonstrating adequate hepatic function helped researchers focus on the next problem, which was thrombocytopenia.

THROMBOCYTOPENIA

Various mechanisms have been proposed for the thrombocytopenia that occurs in liver xenotransplantation, such as 1) tighter binding of porcine von Willebrand factor (vWF) to human platelet glycoprotein Ib (GpIb) causing aberrant platelet activation, 2) increased platelet sequestration and phagocytosis by porcine Kupffer cells as well as non-function of the "do not eat me" signal due to incompatibility between porcine CD47 and human SIRPalpha, 3) platelet consumption by porcine liver sinusoidal endothelial cells via asialoglycoprotein receptor-1, and 4) failure of porcine tissue factor pathway inhibitor to inhibit human tissue factor, causing aberrant activation of the coagulation cascade [11], [38–41] (**Figure 1**).

Burlak et al. demonstrated that thrombocytopenia was a significant problem when they perfused pig livers with human platelets, showing that 93% of the platelets were removed from the circulation within 15 min [42]. When baboons received orthotopic liver xenografts from GTKO pigs transgenic for CD46, severe thrombocytopenia developed within 1 h (although the recipient baboons survived for several days) [43]. To overcome lethal thrombocytopenia in liver xenotransplantation, Kim et al. used aminocaproic acid and achieved 9 days of survival of GTKO pig livers in baboons [44]. Even though there was no evidence of rejection, and aminocaproic acid treatment was successful at keeping the number of platelets at >40,000/mL, the recipients still died or were euthanized for uncontrolled bleeding [44]. As a result,

efforts to overcome thrombocytopenia and coagulopathy continued in liver xenotransplantation research.

A recent explanation for thrombocytopenia is species incompatibility in platelet glycosylation. Burlak et al. showed that platelet alpha granules contain IgM and IgG antibodies targeting xenoantigens, including Gal, Neu5Gc, and Sda. This leads to thrombocytopenia due to platelet phagocytosis by porcine liver sinusoidal endothelial cells, activation of the immune response against platelets, and tethering of platelets in the xenograft [45].

Kupffer cells also play a role in thrombocytopenia seen in xenotransplantation. Mechanisms such as the Mac-1–β-glucan pathway, as well as CD40–CD40L, and CD18–CD40L interactions, are shown to be contributors to platelet phagocytosis. The Mac-1 (CD11b/CD18)-β-glucan interaction enables pig Kupffer cells to recognize and phagocytose desialylated platelets, with exposed N-acetyl D-glucosamine (β-GlcNac) acting as a binding ligand. This interaction triggers phagocytosis of platelets, leading to their rapid depletion [42]. In addition, CD18, which is a part of integrin heterodimer expressed on pig Kupffer cells, recognizes CD40L on human platelets, which were activated due to the inflammatory and immunological environment created during liver xenotransplantation [11]. CD40L is also upregulated on the surface of platelets following activation. This interaction causes phagocytosis of platelets by pig Kupffer cells. Finally, pig Kupffer cells express CD40, and their interaction with CD40L on human platelets triggers immune activation, leading to the release of proinflammatory cytokines, platelet adhesion, and phagocytosis, all of which contribute to thrombocytopenia [11].

COAGULOPATHY

Transfusion of human coagulation factors and immunosuppressive regimens improved the survival of the liver xenograft, but underlying pathophysiological mechanisms persisted [11]. The underlying mechanisms postulated for coagulation dysfunction include pre-existing anti-nonGal antibodies causing tissue factor expression on pig endothelial cells, cross-species molecular incompatibilities in the protein C pathway, incompatibility between porcine vWF and human platelet GPIb, incompatibility between porcine tissue factor pathway inhibitor and human factor Xa, procoagulant tissue factor expression by xenograft endothelial cells, and direct prothrombinase activity of fibrinogen-like protein 2 in xenograft endothelium [46, 47].

To counteract coagulation abnormalities and thrombocytopenia, Navarro-Alvarez et al. administered exogenous human prothrombin concentrate complex, including factors II, VII, IX, X, Protein C and S or Factor VIIa, to baboons that received liver xenografts from GTKO pigs [48]. Even though the recipients were lost by postoperative day 7, decreased transfusions were required in baboons that received exogenous human coagulation factor concentrate. Moreover, those that received Factor VII showed an increase in circulating platelet counts. There was no thrombotic microangiopathy in recipient baboons receiving human coagulation factors, in contrast to previous studies. With 1) the addition of co-stimulation blockade with belatacept or anti-CD40mAb, 2) selection of CMV-negative pig donors, and 3) exogenous use of human coagulation factors, Shah et al. reported 25 days and 29 days of survival following GTKO pig liver xenotransplantation in baboons, with recovery of low platelet counts after postoperative days 7 or 8 [49, 50]. The remarkable survival of two baboons for almost 4 weeks made it possible to anticipate that liver xenografts might be used for bridging to allotransplantation.

Among these mechanisms, the role of thrombomodulin in regulating the coagulation cascade has also been highlighted as a significant contributor to coagulopathy in xenotransplantation. Thrombomodulin, a key component of the coagulation cascade, acts as a cofactor for thrombin and converts thrombin from a pro-coagulant enzyme to an anticoagulant by activating protein C, which subsequently inhibits clot formation. One of the proposed mechanisms in coagulopathy seen in xenotransplantation is the fact that human thrombomodulin may not effectively interact with porcine thrombin due to structural differences, leading to impaired anticoagulant activity and increased risk of coagulopathy. In particular, the interspecies thrombin-thrombomodulin complex has been shown to inadequately activate human protein C, contributing to impaired anticoagulation and leading to coagulopathy [11].

ETHICAL CONSIDERATIONS

While scientific and clinical progress in xenotransplantation provides a new solution to the organ shortage, it also brings to the forefront a range of ethical considerations that must be carefully navigated. Pigs that are raised for organ transplantation are not kept in traditional husbandry conditions; instead, they are kept in clean biosecure (designated pathogen-free) conditions that minimize the risk of pathogen proliferation. This may possibly cause some psychological insecurity that may be detrimental to animal welfare [51].

The use of animal organs, particularly from pigs, can conflict with religious and cultural beliefs and practices, raising issues of respect, dignity, and the need for sensitivity in the medical community. However, when it is a question of life or death, Judaism allows its believers to violate this rule to save human life [52]. In a theological symposium organized by the International Xenotransplantation Association in 2017, the issue of religious beliefs in relation to xenotransplantation was broadly discussed. In the presence of Christian, Jewish, and Muslim representatives, theological perceptions and perspectives about xenotransplantation, the genetic alterations in the organ-source pig, and their acceptance by individuals were explored towards potential clinical trials [53]. There was no absolute opposition to clinical xenotransplantation.

COMMENTS AND CONCLUSION

Even though major challenges remain to be overcome if liver xenotransplantation is to become successful destination therapy, pig-to-NHP liver survival has been extended to 29 days. Pig liver xenografts may therefore play a life-saving role initially as a bridge to allotransplantation for patients with high risk of mortality [50, 54].

An increase in the survival of experimental liver xenografts has directed more attention to the indications for clinical liver xenotransplantation. There are scarce examples of clinical liver xenotransplantation from diverse sources of animals (**Table 1**). Today, it is unlikely that the use liver xenografts from NHPs would be approved [12–17].

Of the two cases of pig-to-human liver xenotransplantation that have been carried out, one was performed using a wild-type pig liver and, despite the removal of xeno-antibodies by plasmapheresis and wild-type pig kidney perfusion, the level of xeno-antibodies increased rapidly, inducing rejection which limited graft survival to a few hours (**Table 1**). To date, the outcome of the second pig-to-human liver xenotransplant using a 10-gene pig liver has not yet been reported scientifically.

Even though it might be premature, more experience gleaned from clinical experiments on "compassionate" grounds might soon justify initial formal clinical trials of bridging to allotransplantation in selected patients with acute liver failure or "liver xenograft bridge-to-regeneration of native liver" in selected liver cancer patients (primary or metastases) where the native liver remnant is insufficient to sustain life.

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SS wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Potential Infectious Complications in Pig Xenograft Donors and Recipients

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Preclinical and clinical xenotransplantation trials have shown that successful outcomes depend on a number of factors including the prevention of xenozoonoses. Preclinical trials involving pig kidneys and hearts transplanted into various non-human primates have revealed the potential impact of pig pathogens being present in the transplanted organ/tissue, mainly viruses. The concept of "designated pathogen-free donor animals" was developed to ensure elimination of pathogens during the breeding of donor animals to mitigate this occurrence. This is a challenging process as confirmation of presence and absence of some pathogen, in particular for latent viruses, requires a validated armamentarium of direct and indirect tests. The importance of using the correct diagnostic regimen was highlighted during the first pig-to-man cardiac transplantation with both porcine cytomegalovirus (PCMV), also known as porcine roseolovirus (PRV), and porcine circovirus (PCV) detected in the transplanted organ and in the patient. To further improve xenotransplantation and to achieve trials in Europe it is important that we use these data to inform process for diagnostics both in donor and recipients before and after xenotransplantation to ensure safety. As part of this sensitive and specific pathogen detection systems should be validated and readily available.

Keywords: xenotransplantation, preclinical trials, clinical trials, porcine viruses, non-human primates

INTRODUCTION

Xenotransplantation poses a unique risk of transmitting pathogens from donor animal grafts to human recipients [1]. The main concern is to fully identify any pathogen risk in the donor animal and to then apply appropriate testing mechanisms to ensure that the recipient is at low risk. Indeed, to date, no formal risk analysis framework has been applied.

Specialized breeding techniques and surveillance monitoring can eliminate most potential pathogens from donor animals, particularly bacterial and fungal pathogens, which are usually acquired after birth [2]. However, certain viruses, particularly porcine endogenous retroviruses (PERVs) and latent viruses, present significant challenges. PERVs, with a genomic origin, are passed to offspring and are difficult to inactivate [3]. There have been numerous studies characterizing PERV in many potential donor herds and all aspects related to PERV are summarized in [4].

For many specific xenozoonotic viruses such as Hepatitis E (HEV), accredited and commercially available serological and molecular tests are available, but this is not always the case.

Latent viruses, which are avoidable by early weaning (raising piglets without maternal contact) and caesarean derivation, are hard to detect even with sensitive PCR methods due to ongoing latency. The tissue used for testing is not always suitable for detection in this instance.

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With this in mind, this article focuses on viral pathogens in xenotransplantation, what we know about their potential impact on the donor organ/tissue and the recipient and what recommendations can be advised as these are the most challenging for diagnosis and elimination.

XENOZOOTIC VIRAL PATHOGENS AND DIAGNOSTICS

Standard surveillance and monitoring is mainly carried out by veterinary diagnostic laboratories, e.g., Animal Plant and Health Agency (APHA, United Kingdom). It is always asked, what pathogens do we need to exclude, is there a list?

Such a list can be separated into pathogens that are important for pig health and those that might affect human recipients. It is also important to note that this "list" will be dynamic depending on the location of the xenotransplant donor herd, as emerging and endemic viruses can vary according to geographical site. It is advised that this is a role of the sponsor to generate a suitable surveillance strategy covering all relevant pathogens for the region with input from the relevant veterinary authority. Such a strategy must be in place for any donor herd and checkpoints for testing and a risk analysis available as a standard procedure. It is also important to consider the type of diagnostic method that is being used for the surveillance as serological and molecular assays will convey different information.

Recently, a suggested microbiological approach and a designated pathogen-free (DPF) list was published by Fishman [1]. As discussed here, it is also important to also consider pathogens that may be present in the recipient that could affect the outcome and be detrimental to the xenotransplant.

Another article by Noordergraaf et al. clearly describes the challenges of maintaining a DPF donor herd and provides invaluable information on the management and exclusion of pathogens over a period of 10 years. Recommendations from their experience emphasize the need for frequent testing and also the importance of caesarean derivation [5].

Key are specific and sensitive assays - which can exclude xenozoonotic viruses - necessary to ensure that donors are not becoming a source of infection for a recipient.

Herein, is the challenge in that many of these are not available. There is a plethora of assays utilized for pathogen detection described in the literature which have been optimized, however, none are fully validated.

Essentially, testing prior to xenotransplant should be carried out by a laboratory with the relevant expertise and validated diagnostic methods. The immediate focus, assuming all other pathogens not permitted in a DPF herd have been proven absent, should be on xenozoonotic viruses. Normally, this would involve molecular analysis for presence of nucleic acid in addition to the presence or absence of antibody.

This also raises the question of what samples should be taken and stored from the donor and recipient. Should we also consider re-testing of organs from the donor post-harvest or organs/tissues to identify any possible risk? Baseline blood and tissue samples must be taken and stored in addition to a biopsy from the proposed organ for xenotransplant.

IS THERE EVIDENCE OF TRANSMISSION OF PORCINE VIRUSES IN PRE-CLINICAL LARGE ANIMAL TRIALS?

Most of the available evidence on viruses in addition to PERV, exists for porcine cytomegalovirus (PCMV), also known as porcine roseolovirus (PCMV/PRV). While there is no conclusive evidence that PCMV/PRV infects human cells *in vitro*, transmission of PCMV/PRV to xenotransplant recipients can have harmful effects [6, 7]. High virus loads in transplanted tissues and associated health issues have been noted, particularly in baboons receiving PCMV/PRV-positive transplants [8]. A consumptive coagulopathy was observed in pig-to baboon kidney xenotransplantation, and an association of PCMV/PRV indirectly suggested by its absence in NHPS receiving organs from PCMV/PRV free donors [2]. A possible mechanism is the observed increase of porcine tissue factor in an *in vitro* model of primary porcine aortic endothelial cells infected with PCMV/PRV, providing a potential link with PCMV/PRV [9]. The role of PCMV/PRV on graft survival was demonstrated by a recently published study in an orthotopic heart transplantation model [7]. Baboon recipients of a PCMV/PRV-positive donor heart developed elevated IL-6, TNF-α and tPA-PAI-1 complex levels pointing to an interference with the cytokine and coagulation system.

Recently, circoviruses have garnered interest in xenotransplantation due to their potential for transmission, as demonstrated in a large animal trial [10]. Although early weaning can be utilized, for full exclusion, caesarean derivation has repeatedly been proposed as the highest standard, albeit it is inconsistent for PLHV [11].

AN APPROACH TO PREVENTION OF TRANSMISSION OF EXOGENOUS AND ENDOGENOUS VIRUSES IN XENOTRANSPLANTATION

Most infectious viral pathogens can be excluded via standard surveillance and monitoring which is mainly carried out by veterinary diagnostic laboratories, e.g., Animal Plant and Health Agency (APHA, United Kingdom). In the large animal trials, strategies such as early weaning have shown some success in improving graft survival by eliminating PCMV/PRV [6, 9]. Over 200 human patients have received pig tissues for treating various conditions, such as diabetes (islet cells), hemophilia (porcine factor VIII), burns (pig skin), and neurological diseases (neuronal cells) [12]. Most of these trials did only use a low number of pig cells and/or a short duration of *ex vivo* perfusion, therefore, microbiological safety could not be tested reliably. A lack of appropriate microbiological tests did add to the challenge.

The first transplantation of a large vascularized organ into a human occurred in January 2022, involving a heart from a genetically modified pig [13]. Despite extensive genetic modifications and testing, PCMV/PRV transmission was missed, and may have contributed to the course of the patient. Recent pig kidney transplants in brain-dead individuals were terminated too early to confirm viral transmission [14, 15].

Single and double stranded DNA and RNA viruses such as member of the families of the *Adenoviridae, Anelloviridae*, *Astroviridae*, *Caliciviridae*, *Circoviridae*, *Parvoviridae*, *Reoviridae*, *Picornaviridae* and others are part of the pig virome [16]. Some viruses are typically found in pig livestock, such as porcine circoviruses (PCVs), African swine fever virus (ASFV), porcine parvoviruses (PPV), and pseudorabies virus (PrV) [17]. Swine influenza virus (SIV) causes a highly contagious viral infection [18]. So far, swine flu has only been described in a few patients usually with close contact, person-to-person infection has not been seen. The potential, however, was clearly demonstrated by the rapid spread of H1N1 in the human population and became a pandemic, causing 60 million cases, and 12,500 deaths in the United States (https://www.cdc.gov/flu/pandemic-resources/2009-h1n1-pandemic.html).

Prevention of these and other pig viruses is dependent on realiable methods of detection, and successful elimination strategies.

Vaccination does play a role in the elimination of some pathogens affecting pig livestock. From a theoretical perspective, different elimination strategies can be used alone or in combination: Caesarean delivery and early weaning, or treatment with antiviral drugs. The "designated pathogen free" status must be confirmed by repeated testing, and use of strict isolation to prevent new introduction. Importantly, for latent infections, confirmation of absence relies on tests measuring the host immune response (e.g. serology).

All the measures described above will also eliminate most if not all remaining bacterial, fungal and parasitic infections. As with allotransplantation, the risk of infection is related to exposure and the impact of immunesuppression. The main route of prevention is in robust screening of the donor animals.

POST XENOTRANSPLANTATION SCREENING OF RECIPIENTS – KEY CONSIDERATIONS

Currently, guidance by the FDA only discusses PERV testing in any detail (FDA 2016). As such, the early studies from xenotransplant recipients focused on the detection of PERV transmission [19].

In the last years numerous PCR-based and immunological assays have been developed to screen donor pigs for potentially zoonotic or xenozoonotic microorganisms. Similar methods are being used in preclinical trials and have been employed for use in patients in the case of clinical trials. Theoretically there is no need to test the recipients for microorganisms which were absent in the donor pig. However, in the case that viruses or other microorganisms were present in the donor pig at very low quantities and below the detection limit of the diagnostic assays used, additional screening of the patient at certain time point after the transplantation is advisable.

As PCR has limited coverage and cannot detect unknown pathogens, it is possible to use NGS and other sequencing technology to identify pathogens present in recipient samples. Meta-genomic NGS (mNGS) does this for entire genomes, while targeted NGS (tNGS) performs this for targeted regions of pathogen DNA or RNA. However, this is not as rapid a method, costs are high and standardization is necessary along with expertise to interpret the large data sets.

For some specific viruses such as PERVs, repeated testing as advised by the FDA (2016) is recommended even if donor animals with inactivated PERV are used [20].

In the recent pig to human cardiac xenotransplantation study, initial observations indicated the presence of PCMV/PRV and PCV in the patient [13]. This does not necessarily mean that the patient is experiencing infection, as it is well known that porcine cells are shed in to circulation and viral DNA/RNA nucleic acid can be detected as a result. Likewise, the use of cell-free preparations from samples does not guarantee that no porcine viral material is present [21]. Further investigation indicated that the xenotransplanted heart was positive for PCMV/PRV as was the donor animal [22]. Viral detection vs. replication and/or productive infection requires expert interpretation and appropriate range of diagnostics.

Fischer et al. described a protocol for combination testing for PCMV/PRV in donor animals to differentiate between active, latent and non-infected animals [23]. The further development and validation of methods such as these are paramount in moving forward in the clinic.

If the scenario does arise that a virus is detected in the patient, anti-virals are available but clinicians must be aware that porcine viruses respond differently, and certain options may not be suitable due to the high dosage required for an effect. For example, ganciclovir prophylaxis is of limited efficacy for PCMV/PRV compared to foscarnet and cidofovir [18].

At this moment in time, there are still some challenging questions. Donor animal samples should be correctly stored but it is not clear how long these should be held and if it is the responsibility of the sponsor. Baseline samples must be retained for both donor and recipient.

What should the approach be for monitoring recipients? What will give us confidence in the pre-xenotransplant diagnostic data from the donor animal? This again supports the need for validation of diagnostic assays and communication with experts in the field to assure the correct interpretation.

More clinical data is required and until this is achieved, it is important to have increased levels of recipient monitoring in the presence of high levels of immunesupporession and in the event of adverse events associated with potential infection.

CONCLUSION: RECOMMENDATIONS FOR CLINICAL TRIALS FOR DONOR AND RECIPIENT SAFETY

Some valuable lessons have been learned from the cardiac xenograft studies in the US and in NHP studies in terms of diagnostics for viral pathogens in donors.

To proceed with clinical investigations in Europe, there are some gaps in the knowledge that require to be addressed. Based on evidence to date, these recommendations should form a baseline for any xenotransplantation process.

1. Future xenotransplantations should use pigs free of potentially zoonotic viruses, achievable through sensitive virus detection and elimination strategies [24].
2. Validated assays must be used for xeno-specific pathogens and take into account the latency that may be associated with porcine herpesviruses.
3. Development of SOP for sampling and testing pre and post xenotransplantation should be developed to permit consistency and reproducibility of assays.
4. Training in compliance, sampling, archiving and diagnostics could also be considered to provide confidence in laboratory diagnostics.
5. Regular follow up of any recipient in a trial using diagnostic tools and the appropriate analysis and interpretation.

Despite efforts to screen donor pigs, unknown pathogen transmission cannot be entirely ruled out. In addition, diagnosing certain porcine pathogens requires complex interpretation and multiple testing reviewed by experts in this field. In this case, a risk assessment and strategy is required in the event of signs of infection in a recipient with early intervention with antivirals and increased monitoring protocols.

Thorough preparation and training of the transplantation team is crucial to ensure xenotransplantation safety [1, 12, 25].

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Monitoring for PERV Following Xenotransplantation

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Porcine endogenous retroviruses (PERVs) are integrated in the genome of all pigs. PERV-A, PERV-B and PERV-C can be released as infectious virus particles and PERV-A and PERV-B can infect human cells in culture. PERV-C does not infect human cells, but high-titer recombinant PERV-A/C can infect them. Retroviruses are able to induce immunosuppression and/or tumors in the infected host. Numerous methods have been developed to study PERV in donor pigs. No PERV infections were observed in infection experiments as well as in preclinical and clinical xenotransplantation trials. Despite this, several strategies have been developed to prevent PERV infection of the recipient. PCR-based and immunological methods are required to screen xenotransplant recipients. Since the proviruses are integrated into the pig genome, PERV infection has to be distinguished from microchimerism, e.g., the presence of pig cells in the recipient, which is common in xenotransplantation. Sensitive PCR methods using pig short interspersed nuclear elements (SINE) sequences allow to detect pig cells easily. Virus infection can also be detected by an increase of viral genomic or mRNA in human cells. The method of choice, however, is to screen for specific antibodies against PERV using different recombinant PERV proteins, purified viruses or peptides.

Keywords: virus safety of xenotransplantation, retroviruses, porcine endogenous retroviruses (PERVs), PERV-C, vaccine

INTRODUCTION

Xenotransplantation may be associated with the transmission of pathogenic pig viruses. The recent report on the first patient receiving a pig heart in Baltimore, Maryland, United States, underlined the importance of virus safety in the context of xenotransplantation. The porcine cytomegalovirus, which is correctly defined as a porcine roseolovirus (PCMV/PRV), was not detected in the xenotransplant due to inappropriate detection methods, but in the transplanted organ the virus was massively replicating due to the absence of the porcine immune system and this contributed to the cascade leading to the death of the patient [1]. Whereas PCMV/PRV and other viruses such as the zoonotic virus hepatitis E virus (HEV), which may induce a disease in the recipient, can be easily eliminated by selection of virus-negative animals, early weaning, colostrum-deprivation, Cesarean delivery or embryo transfer [2], this is not possible with PERVs, since their proviruses are integrated

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Abbreviations: AIDS, acquired immune deficiency syndrome; CRISR/Cas, clustered regularly interspaced short palindromic repeats; CRISPR associated; ddPCR, droplet digital PCR; ELISA, enzyme-linked immunosorbent assay; FeLV, feline leukemia virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HERVs, human endogenous retroviruses; HEV, hepatitis E virus; HIV, human immunodeficiency virus; KoRV, koala retrovirus; LINE, long interspersed nuclear element; MuLV, murine leukemia virus; PCR, polymerase chain reaction; PERV, porcine endogenous retrovirus; PCMV/PRV, porcine cytomegalovirus/porcine roseolovirus; RT, reverse transcriptase; SINE, short interspersed nuclear elements.

in the genome of all pigs. Therefore, PERVs pose a special risk for xenotransplantation using pig cells or organs: Many retroviruses are able to induce immunodeficiencies and tumors in the infected host. Not only the human immunodeficiency virus (HIV) induces severe immunodeficiencies, but also viruses closely related to PERV such as the murine leukemia virus (MuLV), the feline leukemia virus (FeLV) and the koala retrovirus (KoRV) induce immunodeficiencies in addition to leukemias and lymphomas in the infected host. Since there are excellent reviews available, describing the biology, the detection of PERV in donor pigs and the strategies how to prevent PERV transmission to the recipients [3–6], I will give only a short summary on these topics and concentrate on monitoring for PERV infection following xenotransplantation.

BIOLOGY OF PERVS

Retroviruses are characterized by their ability to integrate their RNA genome using the viral enzymes reverse transcriptase and integrase as a DNA copy into the genome of the infected cell. This integrated DNA copy is called provirus. Endogenous retroviruses are the result of integration of proviruses into the oocyte or the sperm cell and consequently, they are present in all cells of the developing organism. PERV-A and PERV-B are present in the genome of all pigs, whereas PERV-C is present in most, but not all pigs. PERV-A and PERV-B are able to infect human cells in culture and therefore pose a risk for xenotransplantation, whereas PERV-C infects only pig cells [5]. However, PERV-C can recombine with PERV-A and acquire so the receptor binding site for the receptor of PERV-A. PERV-A/C can therefore infect human cells and replicate with higher titers compared to the paternal PERV-A. PERV-A and PERV-B infect mainly human tumor cells [7–9], reports concerning the infection of primary cells are rare [9, 10].

DETECTION IN DONOR PIGS

PCR and real-time PCR methods can be used to detect PERV proviruses in the genome of pigs. Droplet digital PCR (ddPCR) allows to quantify the average number of proviruses in the genome of one cell. The copy number depends on the pig breed. In European pigs the copy number is around 60, in Asian pigs the copy number is slightly lower (for review see [11, 12]). To analyze the expression of PERVs at the mRNA level, reverse transcriptase (RT) real-time PCR may be used. To detect the expression at the protein level, immunofluorescence, immunoperoxidase assay or immunohistochemistry using specific sera against viral proteins may be applied. Electron microscopy and measurement of RT activity can be applied to measure the release of virus particles. Infections assays can be used to detect infectious virus particles. PERVs able to infect pig cells will be detected if pig cells are used in these assays; PERVs able to infect human cells will be detected if human cells are used. Details of the mentioned detection methods are described in [13–21].

STRATEGIES TO PREVENT TRANSMISSION

Trans-species transmissions of retroviruses in the sense of infection are well known [2, 22], and the AIDS pandemic is the most disastrous example of a transmission of a zoonotic retrovirus to humans [23]. PERV itself is the result of trans-species transmission of retroviral sequences from rock hyrax, lesser Egyptian jerboa and rodents into pigs [24, 25]. Until now, PERV infections were not observed in all preclinical trials transplanting pig organs into NHPs [26–33]. No PERV infection was observed in first clinical trials transplanting pig cells and organs into human patients, too [26, 34–40]. Furthermore, PERV infection was not observed in infection experiments with small animals as well as with non-human primates under strong immunosuppression [33]. Despite this, numerous strategies to prevent transmission including selection of PERV-C-free animals using PERV-C-specific detection methods to prevent recombination with PERV-A, antiretroviral drugs which are partially also used against (HIV [41, 42], and RNA interference using siRNA [43–47] were developed. Furthermore, vaccines on the basis of neutralizing antibodies against the transmembrane envelope protein p15E and the surface envelope protein gp70 of PERV were generated [48–50]. However, these vaccines could not be tested due the absence of an animal model of PERV infection. Therefore, similar vaccines were developed against the closely related FeLV and it was shown that the vaccine prevented leukemia outbreak in cats infected with FeLV after immunization [51]. Best results were obtained by genome editing. After the failure to inactivate all PERVs using zinc finger nuclease [52], the application of CRISPR/Cas resulted in successful inactivation of all integrated PERVs by deletions in the reverse transcriptase (RT) gene *in vitro* [53] and *in vivo* [54]. However, until now it is unclear whether this inactivation is necessary because - as described in the beginning of this chapter – there is no evidence of PERV transmission to date. Furthermore, there may be off target effects of CRISPR/Cas, and it will be difficult to breed these animals to large colonies. It was shown that CRISPR/Cas treated pig cells are still able to release intact virus particles [55], which, however, should contain viral genomic RNA with an inactivated RT. It is likely that these particles can perform entry into human cells because they carry functional envelope proteins in their envelope. However, due to the inactivated RT they cannot integrate into the genome of the target cell. Since some human cells express RT, either from LINE sequences [56] or from human endogenous retroviruses (HERVs) [57], it cannot be excluded that these RT rescue PERV facilitating reverse transcription and integration. However, it is unlikely that the inactivating mutation in the PERV RT can be repaired or that a recombination between LINE or HERV-RT and PERV-RT is taking place to rescue the virus completely.

DETECTION IN THE RECIPIENT

To screen PERV in the recipient of pig xenotransplants, PCR-based and immunological methods can be used. The use of PCR methods to screen for PERV proviral sequences is difficult, because these sequences are part of the pig genome. Pig cells will be found in all

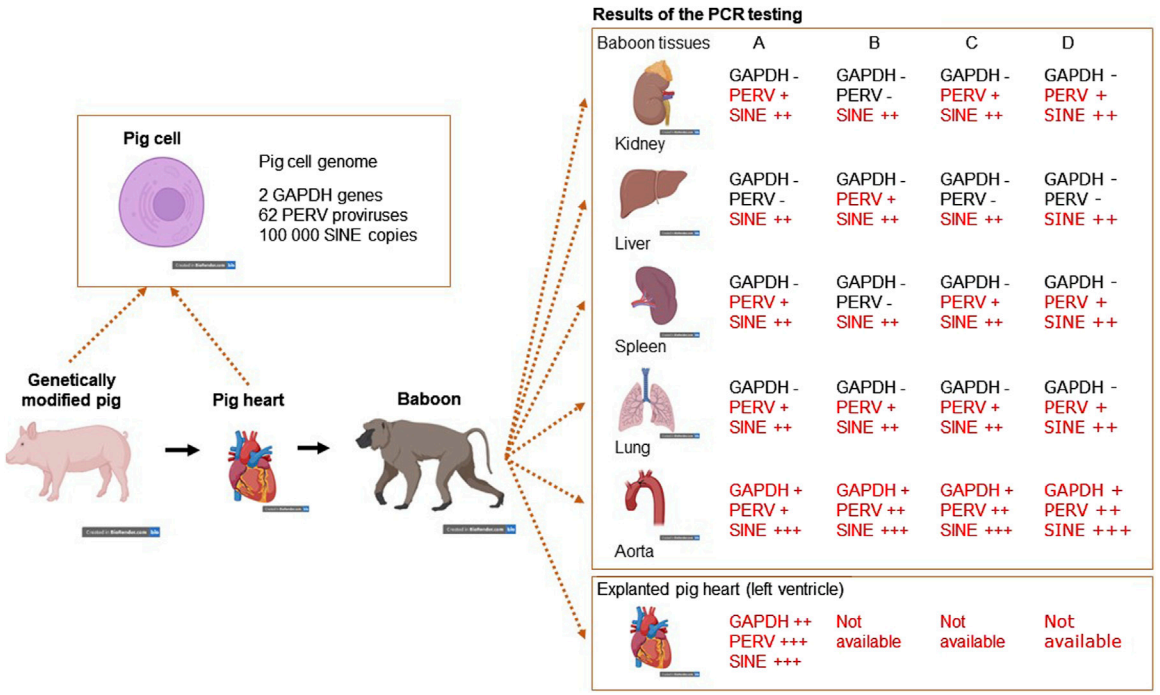


FIGURE 1 | Detection of microchimerism in organs from four baboons (baboon A – survival time 195 days, baboon B – 194 days, baboon C – 26 days, and baboon D – 50 days) after transplantation of a heart from a genetically modified pig. DNA from kidney, liver, spleen, lung and aorta as well as from the explanted pig heart were screened for the presence of porcine GAPDH (2 copies in the pig genome), PERV (approximately 60 copies) and SINE (more than 100,000 copies) using real-time PCRs. Whereas GAPDH as pig marker was found only in the aorta, which is partially of pig origin and in the explanted heart, PERV was found in all organs with exception of the livers from baboons A. C and D. The SINE PCR detected pig cells in all organs analyzed, indicating the presence of microchimerism (This is the Graphical Abstract of publication [59]).

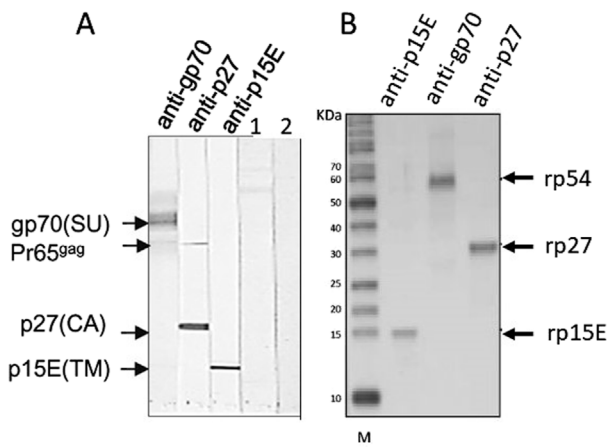


FIGURE 2 | (A) Western blot analysis using gradient purified virus particles separated on an SDS-PAGE and goat sera raised against the recombinant surface envelope protein gp70 (SU) of PERV, recombinant capsid p27 (CA) and recombinant transmembrane envelope protein p15E (TM). The sera detect the corresponding viral proteins as well as the precursor molecule of Gag, $Pr65^{gag}$. In the lanes 1 and 2 sera from patients receiving pig islet cells in the Argentina trial were tested negative (published in [34], with kind permission of Elsevier, 240606-011543). **(B)** Western blot analysis using recombinant rp54 as unglycosylated gp70, rp27Gag and rp15E, which is p15E without the membrane spanning region and the endodomain, and the goat sera as in A. M, marker. (published in [63], with kind permission of Elsevier).

organs of the recipient and will interfere with this testing. The presence of donor cells in the recipient, called microchimerism, is common in xenotransplantation as well as in allotransplantation and pregnancy (for review see [58]). To make sure that the detected proviruses are part of the genome of pig cells, a PCR was developed which detects pig sequences, the so called short interspersed nuclear elements (SINE) sequences [59]. These sequences are found more than 100,000 times in the genome of pigs and this high copy number makes it easy to detect pig cells (**Figure 1**). When we screened baboons for PERV, PERV sequences were found in most of the organs tested (there are around 60 PERV copies in the pig genome), GAPDH sequences were rarely found (there are only 2 copies in the pig genome), but SINE sequences (more than 100,000 copies per genome) were found in all baboon organs tested, indicating microchimerism (**Figure 1**) [59]. Another possibility is to screen for increasing amounts of viral genomic or viral mRNA using a RT real-time PCR or sequencing of RNA, indicating virus replication. Another very effective way of testing for PERV would be looking for spliced env mRNA, which is a prerequisite for the translation of the Env protein and particle release [60, 61]. However, there is the possibility that viral RNA will be produced in pig cells expressing PERV or even releasing PERV. To demonstrate an infection, viral RNA has to be localized in human cells.

Having in mind these difficulties, the best method to detect PERV infection in the recipient is to detect antibodies against PERV as an indirect sign of virus infection. Antibody screening is a

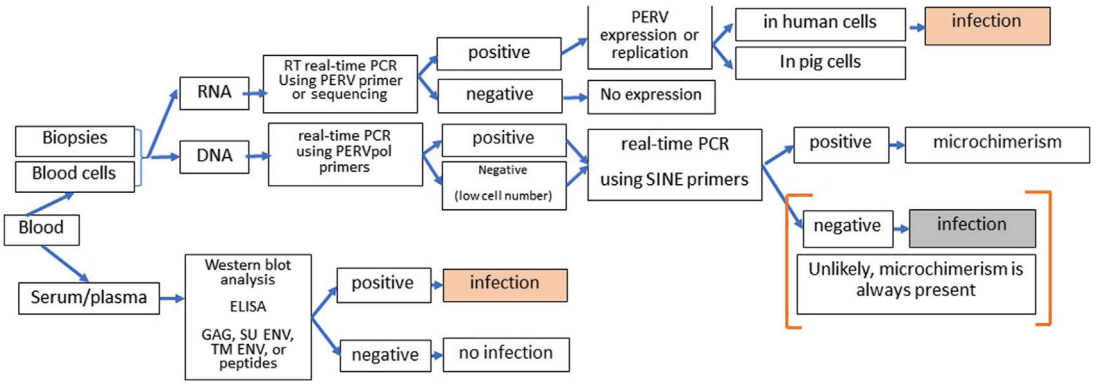


FIGURE 3 | Strategy of screening for PERV infection in xenotransplantation recipients. DNA and RNA from blood cells or biopsies can be tested by PCR or RT-PCR for the presence of PERV proviruses and PERV expression. Since microchimerism is always present in xenotransplanted individuals, detection of PERV sequences in the DNA will always be associated with pig cell. When increasing amounts of PERV genomic and mRNA were observed, it has to be assured that virus replication takes part in human cells to diagnose PERV infection of the recipient. The presence of antibodies against two or more PERV antigens (GAG, capsid protein p27; SU ENV, surface envelope protein gp70, TM ENV, transmembrane envelope protein p15E, or corresponding peptides) using Western blot assays or ELISA clearly indicates an PERV infection. Absence of immune reaction means absence of infection approximately 3 to 4 weeks before.

common method to detect retrovirus infection and is widely used to screen for an infection with HIV [62]. There are two main methods to detect PERV-specific antibodies, ELISA or Western blot analysis. Recombinant PERV proteins or purified virus particles can be used as antigens in Western blot analyses [19, 28, 34, 38] (**Figures 2A, B**). The advantage of Western blot analyses when using recombinant proteins is that the specific band can be accurately identified based on size, whereas false-positive reactions can be expected in ELISA when using recombinant proteins due to insufficient purity of the proteins produced in bacteria. Using lysates of highly purified virus preparations also allows to detect antibody responses against different viral proteins (**Figure 2A**). An ELISA can be performed using synthetic peptides corresponding to immunodominant epitopes of the viral proteins [19]. Such a immunodominant epitope was detected in the transmembrane envelope protein of several retroviruses [19]. It is very important to use two or three PERV proteins, for example the core protein p27Gag, the surface envelope protein gp70 and the transmembrane envelope protein p15E or corresponding synthetic peptides (**Figure 2**). It is not recommended to use p27Gag of PERV alone since there are a few human individuals who have antibodies against p27Gag of PERV, despite the fact that they are not infected [19]. The absence of antibodies against other PERV proteins indicates that they are not infected. It remains unclear whether the response against p27Gag in these few individuals is due to cross-reactive antibodies directed against an auto-antigen, a parasite-derived antigen or a related retrovirus [64–67]. False positive antibodies against the core protein p24 of HIV-1 were also common, for example in patients with systemic lupus erythematosus [68, 69].

There is one additional question: Are immunosuppressed individuals who received a pig organ able to mount an antibody response? Since immunosuppressed patients after allotransplantation are able to mount sufficient immune responses after vaccination, it is likely that xenotransplant patients can produce antibodies against PERV proteins [70, 71].

CONCLUSION

There are numerous assays available to screen for PERV infection in the recipient [72]. However, it is not easy since microchimerism, e.g., presence of pig cells in all organs of the recipient, complicates the proof. Therefore, it is important to distinguish between infection of human cells on one hand and proviruses in disseminated pig cells, which will always be present in xenotransplanted individuals, on the other hand. Highly sensitive SINE PCR will easily detect microchimerism. One further method is the detection of increasing amounts of viral genomic or mRNA indicating replication of PERV. However, to prove infection, it should be shown that this replication takes place in human and not in pig cells. The method of choice is the detection of antibodies against PERV using at least two or three recombinant viral proteins or corresponding synthetic peptides or purified virus particles in a Western blot assay or ELISA (**Figure 3**).

AUTHOR CONTRIBUTIONS

JD conceptualized and wrote the article.

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CONFLICT OF INTEREST

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Cytotoxic Responses Mediated by NK Cells and Cytotoxic T Lymphocytes in Xenotransplantation

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Xenotransplantation represents a potential solution to the shortage of organs for transplantation. The recent advancements in porcine genetic modification have addressed hyperacute and acute vascular rejection; however, challenges persist with regard to delayed xenograft rejection. Porcine endothelial cells (pECs) represent a crucial target in the context of xenograft rejection, which is mediated by cytotoxic lymphocytes. It is crucial to comprehend the manner in which human natural killer (NK) cells and cytotoxic $CD8^+$ T lymphocytes (CTL) recognize and target pECs in order to develop efficacious prophylactic strategies against rejection. The objective of the present review is to synthesize the existing knowledge regarding the mechanisms and techniques employed to modulate xenogeneic responses mediated by human NK cells and CTL. We will elucidate recent methodological advancements, debate potential novel strategies, and emphasize the imperative necessity for further research and innovative approaches to enhance graft survival.

Keywords: porcine endothelium, NK cells, $CD8^+$ T cells, xenograft rejection, cytotoxicity

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INTRODUCTION

Numerous hurdles in xenotransplantation need to be overcome to achieve tolerance of the recipient's immune system [1–3]. Hyperacute rejection seems to be resolved due to the availability of knockouts of the major porcine xenoantigens such as αGal [4–6]. Even so, acute vascular and delayed xenograft rejection mediated by both the innate and adaptive immune system remain an obstacle to successful xenotransplantation despite novel systemic immunosuppressive regimens.

The endothelium of a vascularized porcine xenograft represents the initial point of contact with the recipient's blood, which contains a multitude of immune cell subsets. Consequently, a significant objective of contemporary research initiatives is to evaluate the impact of diverse genetic modifications in porcine endothelial cells (pECs) on human immune cells. It was shown that the complement- and coagulation-mediated damage of pECs can be prevented by knocking out the genes of the three major xenocarbohydrates (αGal, Neu5Gc, and Sda), GGTA1, CMAH, and B4GALNT2, respectively [4], by introducing human complement regulators (hCD46, hDC55, and hCD59) [7, 8], and thrombomodulin (hTBM). Overcoming these hurdles of hyperacute and acute vascular rejection, consistent life-supporting function of xenografted hearts for up to 195 days was achieved in a pig-to-non-human primate (NHP) preclinical model with a combination of αGal knockout (GTKO), and transgenic expression of hCD46 and hTBM [9]. Consistent long-term survival was also demonstrated in pig-to-NHP kidney xenotransplantation using 10GE (genetically engineered) source pigs and FDA-approved

immunosuppression [10]. These data provide critical supporting evidence for the safety and feasibility of clinical kidney xenotransplantation, even without CD40/CD154 costimulatory blockade. However, it was not further explored in these models, whether responses mediated by natural killer (NK) cells and cytotoxic $CD8^+$ T lymphocytes (CTL) were sufficiently controlled. Further, pig-to-primate experiments are considered as the best-possible pre-clinical constellation for evaluating xenotransplantation, but it is largely unclear to which extent genetic modifications that have been designed for humans remain purposeful in baboons.

The significance of NK cells in xenograft rejection was first shown in the early 90s by Inverardi et al using an *ex vivo* human-to-rat perfusion model [11]. Subsequently, our research group and others have conducted comprehensive, predominantly *in vitro* investigations into the function of NK cells in pig-to-human xenotransplantation [12–14]. A comprehensive review of the immunological barriers, including strategies of inhibition, was published by Sykes and Sachs in 2022 emphasizing approaches to promote and induce T cell tolerance in xenograft recipients [2]. The goal of the present review is to focus on our current understanding of the mechanisms involved in xenogeneic responses mediated by human cytotoxic lymphocytes (NK cells and CTL), in the light of recent advances in the field of xenotransplantation, and to discuss potential strategies for prevention of xenograft rejection.

RECRUITMENT AND ACTIVATION OF CYTOTOXIC LYMPHOCYTES BY PORCINE ENDOTHELIAL CELLS

Antibody binding, complement deposition, and coagulation proteins activate porcine endothelium leading to the release of pro-inflammatory cytokines such as TNF and IL-6. These cytokines subsequently recruit immune cells to the graft [15, 16]. Activated pECs express E- (CD62E) and P- (CD62P) selectin which bind to L-selectin (CD62L) on human lymphocytes, thereby initiating the rolling phase. Human β_2 integrins, such as LFA-1 (CD11a/CD18), interact with immunoglobulin superfamily members (i.e., porcine ICAM-1) enabling firm adhesion of the lymphocytes [17]. It is of particular significance that the human β1 integrin very late antigen-4 (VLA-4; CD49d/CD29) has the capacity to bind to VCAM-1 (CD106) on pECs [17–19], thereby facilitating NK cell and CTL adhesion. Lastly, it has been demonstrated using transwell assays *in vitro* that transendothelial migration of human leukocytes is dependent on CD99 and the β_2 integrin CD18 [18]. Conversely, homotypic interactions mediated through CD31 (PECAM-1) have been shown to be incompatible in the pig-to-human context [19].

NK cells and CTL mediate anti-pig cytotoxicity through lytic granule release (perforin and granzyme B) or the Fas/FasL and TRAIL pathways, causing apoptotic and/or necrotic death of the target cells [20–25]. While sharing the killing mechanisms, the robust response of NK cells towards target cells can in turn initiate and amplify CTL-mediated cytotoxicity [26, 27]. Both produce cytokines like interferon-gamma (IFNγ) and tumour necrosis factor (TNF) [28]. In the recent *in vivo* studies of pig-to-NHP and pig-to-human (decedent recipients) solid organ transplantation, NK cells were found to be the early producers of IFNγ [29, 30]. An overview of the classical techniques used to evaluate NK cell and CTL xenoresponses *in vitro* and *ex vivo,* as well as an overview of the current cutting-edge -omics techniques, such as single-cell RNA sequencing (scRNA-seq) and longitudinal RNA-seq analyses, is presented in **Box 1**. To assess cell-cell interactions, i.e., lymphocytes adhesion to pECs and transendothelial migration under shear stress, our group has recently established a 3D microfluidic system [31], [Tran T et al., in press].

Natural Killer Cells

In the context of pig-to-human xenotransplantation, incompatibilities in species-specific molecular interactions can either result in the activation of NK cells or prevent it through a lack of inhibitory signals (**Figure 1**). NK cells exert their function via two primary mechanisms (i) antibody-dependent cellular cytotoxicity (ADCC) and (ii) direct cytotoxicity. ADCC is initiated when the NK cell receptor CD16 (FcγRIIIA) binds to human antibodies that are directed against xenoantigens expressed on pig endothelium; (ii) in direct cytotoxicity the lack of recognition ("missing self") of SLA (swine leukocyte antigen) MHC class-I molecules by inhibitory NK cell receptors (CD94/NKG2A, KIRs, and ILT2) [32–34] results in the activation of NK cells. Another immune checkpoint molecule providing inhibitory signals, SIRPα (CD172a), can be expressed on NK cells upon cytokine stimulation [35]. Among different activating NK cell receptors CD2 (LFA-2), NKp44 and NKG2D are involved in both porcine cell lysis and cytokine production [36, 37]. Porcine CD58 may function as an activating ligand for CD2 expressed on human NK cells [38, 39] as evidenced by inhibitory effects of pCD58 blockade on human peripheral blood mononuclear cells (PBMC) cytotoxicity and proliferation in response to porcine cells [40]. The interaction between human NKG2D and porcine ULBP1 (pUBPL1) remains a topic of some debate. The porcine ligand for the activating NK cell receptor NKp44 is still unknown, reported candidates include proliferating cell nuclear antigen (PCNA), heparan sulfate proteoglycans (HSPGs), viral hemagglutinin, MHC class I related chain A (MICA), platelet-derived growth factor (PDGF)-DD, and some HLA-DP molecules [41, 42], however, a widely expressed cellular ligand for NKp44 has remained elusive and controversial. Finally, the impact of glycosylation, in particular sialic acids on the interaction between NK cells and potential target cells needs to be further investigated [43]. The expression of Siglec-7 ligands on target cells can protect them from NK cell-mediated lysis whereas masking of ligands for Siglec-7 and Siglec-9 has been demonstrated to enhance anti-tumour activity of NK cells. This aspect has not yet been evaluated in the context of transplantation.

BOX 1 | Methods to analyse NK and CTL porcine xenoresponses.

***In vitro* (for NK and CTL unless said otherwise)**

Cytotoxicity release assay. Here, effector cytotoxic cells are co-cultured with porcine target cells (either pPBMC or pEC) previously loaded with ^{51}[Cr] or BADTA which after cellular membrane destruction are released to the culture media and quantified.

CD107a degranulation assay. In this flow cytometry-based assay, the degranulation of lytic granule contents by cytotoxic cells upon encounter with porcine target cells is detected by the surface expression of CD107a thanks to the fusion of lytic granule to the cellular membrane. This assay could be coupled to intracellular cytokine detection.

Adhesion assays using fluorescence time-lapse microscopy. Porcine endothelial cell destruction is quantified in real-time upon encounter with cytotoxic cells. This method allows in addition to study cytotoxic cell displacement on a carpet of pEC and type of cell elimination. The next level is achieved by using a 3D microvessel system covered by a monolayer of pEC where cytotoxic cells first adhere to encounter and destroy their targets under flow.

Mix lymphocyte reaction (MLR). This assay is specific for T cells and measures T-cell responses from the recipient (human or NHP) to xenoantigens. It involves co-culturing responder lymphocytes (recipient) with irradiated or Mitomycin C-treated stimulator cells (donor PBMC) to assess proliferation through thymidine incorporation, CFSE or BrdU staining, or cytokine production by ELISpot or ELISA. Xeno-specific T cells proliferate upon encountering antigens on stimulator cells to which they are specifically reactive, such as SLA-1.

Transendothelial migration (TEM) assay. Is used to study the movement of lymphocytes across the endothelial layer. Endothelial cells are cultured in Transwell insert which has a porous bottom that allows immune cells to migrate through it. Lymphocytes migrate into wells containing a chemoattractant or medium and can be quantified once the transmigration is completed.

Antibody-dependent cell cytotoxicity (ADCC). This assay measures the activation of NK cells via the CD16 receptor, leading to target cell lysis when IgG xenoantibodies present in the recipient's sera bind to donor-derived cells. It is performed similarly to the cytotoxicity release assay, where donor cells are labeled beforehand.

Ex vivo

Biopsies. Analysis by histology, immunohistochemistry, or immunofluorescence is performed in some cases both during graft acceptance and rejection. This examination evaluates cellular infiltration, tissue architecture, and marker expression to assess immune responses, inflammation, and graft viability. These analyses offer insights into the mechanisms of graft acceptance or rejection, but they are purely descriptive and do not evaluate functional outcomes.

Analysis of CTL and NK cells functions in blood. Here the same assays of cytotoxic release assay, CD107a degranulation, MLR, ADCC.

Omics era

Bulk and single-cell RNA sequencing. As a starting material, either biopsies of a xenograft or recipient's PBMC are used to measure gene expression across the entire cell population (bulk RNAseq) or for each individual cell within the population (scRNAseq). This allows to identify genes of interest which could be targeted in the xenotransplantation setting.

Spatial transcriptomics of single nucleus/cells. Combines histology and RNAseq. It enables the mapping of gene expression across different regions of a tissue sample, maintaining spatial context. The method can map the immune response at the site of the xenograft. This includes identifying immune cell infiltration patterns and understanding local cytokine and chemokine expression.

Cytotoxic T Lymphocytes

Unlike NK cells, naïve CD8$^+$ T cells require prior stimulation with porcine antigens target cell elimination [44]. *In vitro* data show that human T lymphocytes recognize porcine cells via both direct and indirect pathways [45–49]. Direct recognition entails T cell receptor (TCR) interactions with SLA-I/II and co-stimulatory molecules CD80/86 [48–52], via CD2 and CD28 pathways [53] on pECs (**Figure 2**), whereas indirect recognition involves the presentation of pig antigens by recipient's antigen-presenting cells (APC) [54]. Moreover, *in vitro* studies have demonstrated that the proliferative responses of human T cells are enhanced when both porcine cells and human autologous APC are co-cultured, thereby combining direct and indirect recognition mechanisms [55, 56]. Additionally, indirect presentation was described by the recognition of SLA-I within endothelial cell derived extracellular vesicles [52]. CTL not only recognize SLA-I allelic polymorphisms, but they also require the presence of porcine peptides bound to SLA-I for an efficient xenospecific reaction [57–59]. Consequently, specific T cell clones proliferate and differentiate into effector and then memory cells that react rapidly upon the antigen re-exposure [60]. Co-stimulation can also be provided via pCD86 expressed on porcine cells binding CD28 on both CTL and NK cells [61–64], as well as binding of CD154 (CD40L) and CD40 [65, 66] (**Figures 1**, **2**). It is noteworthy that *in vitro* studies utilizing human and baboon PBMC demonstrated a reduction in T cell responses to GGAT1 KO pig-derived pEC in comparison to wild-type pEC, irrespective of whether the pEC were activated. CD4 and CD8 lymphocytes showed reduced proliferation, cytokine production, and granzyme/perforin content. Nevertheless, the specific mechanisms by which αGal modulates the T cell response remain to be elucidated [67].

Findings in the Recent Studies *In Vivo*

The findings across various animal models demonstrated the complexity and challenges associated with NK and CTL responses in pig-to-primate xenotransplantation. In baboons that received porcine heart transplants expressing either hCD55 or hCD46, without specific T cell depletion, the rejection process occurred rapidly, within 5–7 days, and was characterized by a high prevalence of macrophages and T cells [68, 69]. Similarly, porcine islet transplantation into cynomolgus monkeys and rhesus macaques resulted in infiltration and rejection, which were primarily mediated by T cells, even before macrophage recruitment into the tissues [70, 71]. In the case of porcine cartilage transplanted into cynomolgus monkeys, up to 90% of the infiltrating cells were T cells, with equal amounts of CD4$^+$ and CD8$^+$ T cells, as observed 1 month post-transplantation [72]. Recent studies have demonstrated the involvement of NK cells and a few adaptive CD3$^+$ cells in porcine kidney xenografts transplanted into brain-dead recipients. NK cells and T cells were identified in the glomeruli 54 h post-transplantation, with transcriptome profiling showing increased expression of genes related to IFNγ response, NK cells (*HLA-E* and *FCGR3A/B*), and T cell development (*CD81*) [73]. Longitudinal bulk RNA-seq of kidney xenografts revealed increased IFNγ signaling as early as 12 h post-transplantation, predominantly attributed to infiltrated NK cells [30]. Single-cell RNA-sequencing of recipient's PBMCs revealed two distinct waves of immune response with upregulation of genes associated with NK and T cells. This correlated with increased

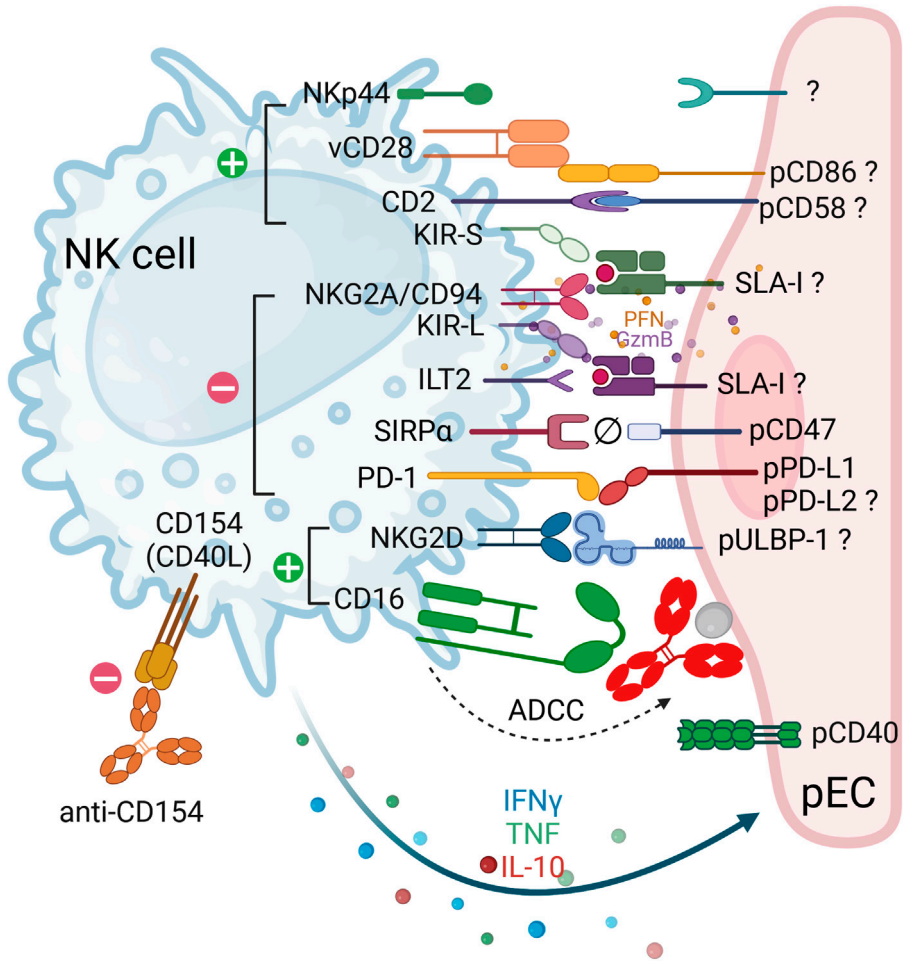


FIGURE 1 | Receptor-ligand interactions between human NK cells and porcine endothelial cells. The cartoon depicts the interactions between human natural killer (NK) cells (blue) and porcine endothelial cells (pEC, pink). In instances where the ligand-receptor pair interaction is functional across species, the corresponding pair is indicated. In cases where the ligand-receptor pair is suspected, or when contradictory data is available, a question mark is added to indicate this. Ligand-receptor interactions that result in either activation or inhibition are represented by green (+) and red (–) circles, respectively. Incompatibility between pCD47 and hSIRPα is symbolized by Ø. The engagement of activating receptors results in the release of lytic granule content (perforins, granzymes and granulysins), which is then followed by the secretion of cytokines (e.g. IFNγ, TNF and IL-10). Co-stimulatory molecules expressed on pEC include CD80/CD86 and PD-L1. NK cells are also capable of expressing these 'co-stimulatory' molecules (CD28 and PD-1), thereby regulating their function. Therapeutic strategies that interfere with the costimulatory pathways include anti-CD40L (anti-CD154), as shown in the figure, anti-CD40 and the soluble CTLA-Ig molecule, among others. Finally, NK cells can be strongly activated by the binding of their CD16/FcgRIIIa to antibodies attached to pEC leading to antibody-dependent cellular cytotoxicity (ADCC).

IFNγ expression in NK cells at 12 and 48–53 h [30]. Similar trends were observed through scRNA-seq analysis on day 38 in the first pig-to-human cardiac xenograft recipient. Dominant changes in immune cell type composition across the time points were observed for monocytes, both $CD4^+$ and $CD8^+$ T cells, and NK cells, accompanied by the upregulation of genes related to NK and T cell activation, and IFN responses. However, it was difficult to pinpoint any single etiology for the xenograft dysfunction [74]. The latest multi-omics analyses of samples from two deceased human recipients of 10-gene-edited pig hearts have revealed two distinct scenarios regarding the administration of thymoglobulin and the duration of cold ischemic time. One of the decedents exhibited a lack of evidence of immune cell activation, whereas the other decedent displayed an increase in T and NK cells in both circulating PBMC and xenograft [75]. This notable rise in lymphocytes in one of the recipients may be attributed to the longer ischemic time of the pig heart and the T cell depletion protocol resulting in the expansion of memory T cells and rapid xenograft injury.

STRATEGIES OF INHIBITION

NK Cells

Based on the regulation of NK cell function predicted by the "missing self" theory and the observation that SLA MHC class I molecules do not appear to interact sufficiently with inhibitory human NK cell receptors, human MHC class I molecules were introduced into pig cells to protect against human NK cytotoxicity

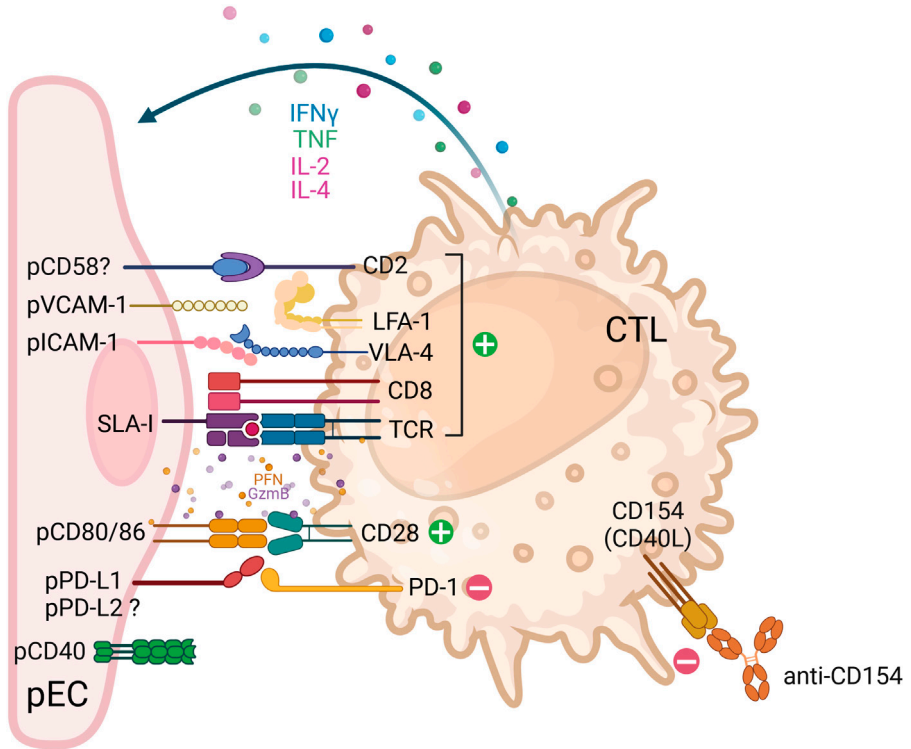


FIGURE 2 | Receptor-ligand interactions between human cytotoxic T lymphocytes (CTL) and porcine endothelial cells. The cartoon depicts the interactions between cytotoxic T lymphocytes (CTL) (CD8+ T cells, orange) with porcine endothelial cells (pEC, pink). In instances where the ligand-receptor pair interaction is functional across species, the corresponding pair is indicated. In cases where the ligand-receptor pair is suspected, or when contradictory data is available, a question mark is added to indicate this. Ligand-receptor interactions that result in either activation or inhibition are represented by green (+) and red (-) circles, respectively. The engagement of activating receptors results in the release of lytic granule content (perforins, granzymes and granulysins), which is then followed by the secretion of cytokines (e.g. IFNγ, TNF, IL-2 and IL-4). The term 'costimulatory pathways' was first employed in the context of T cell immunology following TCR engagement (signal 1). The term is used to describe the molecules that are required to further activate or inhibit T cells (signal 2). Such molecules include CD80/CD86 and PD-L1. It is noteworthy that the co-stimulatory CD28 is a dual-function molecule on CTL, capable of binding to CD80/CD86 on pEC, thereby providing activation or to CTLA-4 on helper CD4 T cells, thereby triggering an inhibitory signal. Therapeutic strategies that interfere with the costimulatory pathways include anti-CD40L (anti-CD154), as shown in the figure, anti-CD40 and the soluble CTLA-Ig molecule, among others. Abbreviations: ADCC: antibody-dependent cellular cytotoxicity; CD154: also known as CD40L; CD16/FcgRIIIa: Fc gamma Receptor IIIa; CD2: also known as lymphocyte-function antigen-2 (LFA-2); CTL: cytotoxic T lymphocyte; CTLA-4: cytotoxic T-lymphocyte associated protein 4, also known as CD152 molecule; GzmB: granzyme B; IFNγ: interferon-gamma; IL: interleukin; ILT2: immunoglobulin-like transcript 2, also known as leukocyte immunoglobulin like receptor B1 (LILRB1); KIR: killer cell immunoglobulin-like receptor, -L – inhibitory, -S - activating; LFA-1: lymphocyte function-associated antigen 1, also known as CD11a molecule; NK: natural killer; NKG2A: also known as killer cell lectin like receptor C1 (KLRC1); NKG2D: also known as killer-cell lectin-like receptor K1 (KLRK1); NKp44: natural cytotoxicity triggering receptor 2, also known as NCR2 and CD336 molecule; p: porcine; PD-1: programmed cell death 1 also known as CD279 molecule; PD-L1/2: programmed cell death 1/2 ligand, also known as CD274/CD273; PFN: perforin; SIRPα: signal regulatory protein alpha also known as CD172a molecule; SLA-I: swine leukocyte antigen-1; TCR: T-cell receptor; TNF: tumour necrosis factor; ULBP-1: UL16 binding protein 1; vCD28: variant of CD28; VLA-4: very late antigen-4, integrin formed by alpha4/beta4 subunits (CD49d/CD29 molecules).

[12, 13, 76–78] (**Table 1**). For example, HLA-E is a low polymorphic, non-classical HLA class I molecule and a major ligand for the inhibitory receptor CD94/NKG2A, which is expressed on a subset of NK cells. Thus, expression of HLA-E on pig endothelial cell at least partially prevents direct human NK cytotoxicity. This finding was confirmed in *in vitro* studies [79, 80] and in *ex vivo* perfusion studies of porcine limbs, hearts and lungs with human blood [8, 76, 81]. In addition, decreased proliferation of both human T and NK cells was demonstrated *in vitro* in response to fibroblasts derived from GTKO pigs expressing HLA-G1$^+$. Furthermore, transplantation of islets from GTKO/HLA-G1$^+$ pigs into diabetic mice resulted in restored normoglycemia [77]. Several other studies have confirmed the partial inhibition of NK cell cytotoxicity, but also of adhesion, by HLA-G1 expressed on pEC *in vitro* [96–98], including soluble HLA-G1 [99]. Finally, higher levels of protection have been achieved by co-expressing HLA-E and HLA-G1 *in vitro* assays, primarily by stabilizing HLA-E expression [82, 83]. However, complete protection from xenogeneic NK cytotoxicity has not yet been achieved, probably due to the heterogeneity of the inhibitory NK cell receptor repertoire.

The role of αGal in recognition of porcine target cells by human NK cells was extensively reviewed in 2017 [12]. In short, human NK cytotoxicity against αGalKO pEC in the absence of Abs was equivalent to that in αGal expressing cells. On the other hand, interactions between αGal and the activating NK cell receptor CD161 remains a matter of debate [100–102]. The deletion of αGal might result in the recognition of terminal epitopes and

TABLE 1 | Strategies of inhibition of human cytotoxic lymphocytes tested to date.

Genetic modification	Model	Inhibited lymphocytes	Reference
HLA-E	*Ex-vivo* perfusion of pig hearts	NK cells	[76]
	In vitro		[79, 80]
	Ex-vivo perfusion of pig limbs		[8]
	Ex-vivo perfusion of pig lungs		[81]
Co-expressing HLA-E and HLA-G1	*In vitro*		[82, 83]
pULBP1 KO	*In vitro*		[84,85]
Beta-2 microglobulin (KO B2M)/SLA-I low	*In vitro*	$CD8^+$ T cells	[86]
Membrane-bound human FasL and human decoy Fas	*In vitro*; pig-to-rat islets Tx		[21, 87]
Porcine cellular FLICE inhibitory protein (c-FLIP)	*In vitro*; pig-to-rat TG PAEC Tx under kidney capsules		[88]
HLA-G1	*In vitro*; pig-to-mouse islets Tx	T cells, NK cells	[77]
hCD152 (hCTLA4-Ig)	*In vitro*; pig-to-mouse TG PAEC Tx under kidney capsules	T cells, NK cells	[64, 89]
	In vitro; pig-to-rat skin Tx	PBMC	[90]
LEA29Y[a]	*In vitro;* pig-to-mouse islet Tx	T cells	[91,92]
hPD-L1	*In vitro*	T cells, NK cells	[93, 94]
	In vitro; Ex-vivo kidney perfusion	T cells ($CD4^+$ and $CD8^+$)	[95]

[a]*LEA29Y is an affinity-optimized derivative of hCTLA4-Ig.*

N-acetyllactosamine (NAcLac) [100] by NK cells expressing CD161, however, the significance of such neoantigens needs further investigation. Immortalized pEC with gene KO for the enzymes of the three major xenoAg sugars, GGTA1, CMAH and B4GALNT2, with or without deletion of SLA-I α-chain/β-2, strongly induced degranulation of IL-2-activated human NK cells [103]. The results indicate that other so far unidentified activating ligands of carbohydrate and/or protein nature, are involved in human anti-pig NK cytotoxicity. The question of the exact ligands on porcine cells stays unsolved. However, some of these unidentified ligands can be masked by dextran, an analog of heparan sulfate proteoglycan, which is a key molecule for the anticoagulant and anti-inflammatory properties of the resting (non-activated) endothelium, providing partial protection against human NK cytotoxicity [104].

Human NK cell receptors NKp44 and NKG2D are activated by ligands present in pECs [37] and porcine chondrocytes [105]. Although porcine ULBP1 has been confirmed as a ligand for human NKG2D, its removal did not result in complete inhibition of NK cell cytotoxicity, indicating the potential involvement of additional ligands [106]. Using CRISPR technology, more recent findings demonstrated that pULBP1 gene knockout in porcine endothelial cell lines or transgenic pigs was not sufficient to fully suppress NK cell degranulation and cytotoxicity [84, 85].

Some other alternative and indirect methods of NK cell inhibition were explored *in vitro*. Controversial data have been reported on the protective effect of FasL expression on porcine endothelial cells [21, 87, 107]. An effective strategy to mitigate macrophage responses is the transgenic expression of CD47 on porcine cells, which acts as a "don't eat me" signal via the receptor SIRPα and inhibits phagocytosis [108–110]. Of note, CD47 was present in the 10GE pig hearts used in the first pig-to-human xenotransplantations. It appears that it did not entirely prevent macrophage responses, as CD68 staining demonstrated clusters of macrophages on POD34. However, CD47 gene expression in the heart decreased over time, as other transgenes (HO1, CD46, and DAF) also showed lower expression, raising the question of how effective and robust the genetic modifications were [74]. Since NK cells can also express SIRPα they could potentially be inhibited via this pathway. Indeed, expression of CD47 on HLA-deficient target cells inhibited SIRPα-expressing NK cells *in vivo* in a mouse model and *in vitro* using a CD47 transduced NK target cell line (K562) [35]. These recent results need to be confirmed in the pig-to-human and pig-to-NHP settings.

Cytotoxic T Lymphocytes

As mentioned above, T cells recognize porcine cells directly restricted by MHC molecules; therefore, elimination of SLA class I expression on pig cells should protect from $CD8^+$ T lymphocyte-mediated lysis. Indeed, cells obtained from engineered pigs knocked down for beta-2 microglobulin (KO B2M) and expressing an SLA-I^{low} phenotype were less capable in triggering proliferation of human PBMC *in vitro*, which was mainly due to the non-responsiveness of $CD8^+$ T cells. However, no difference in cytotoxicity exerted by human PBMC or purified NK cells was observed [86]. Alternatively, as shown in a recent *in vitro* study, silenced expression of SLA-I and -II on porcine kidney epithelial cells resulted in weak CTL reactivity and cytokine secretion but, surprisingly, did not trigger NK cell degranulation [111]. Therefore, further investigation is required to gain a deeper comprehension of the impact of SLA knockouts or silencing on CTL and NK cell responses. This is crucial to prevent the potential exacerbation of the missing-self problem.

One of the ways to prevent delayed xenograft rejection (DXR) orchestrated by adaptive immunity is the administration of immunosuppressive therapy (see below), including blocking co-stimulatory molecules of T cell – APC interactions (i.e., CD80/CD86-CD28) with CTLA4-Ig (Abatacept) or its affinity-optimized derivative LEA29Y (Belatacept) [112]. To circumvent the drawbacks of systemic immunosuppression, these inhibitory molecules have been expressed transgenically in pig cells with the objective of enabling local protection of the xenograft from CTL [91]. It was shown that serum from LEA29Y [92] and hCTLA4-Ig [90] transgenic pigs reduced the proliferation of

T cells in a mixed-lymphocyte reaction compared to wild-type (WT) controls. Another similar approach to protect xenograft is by expression of human programmed death-ligand 1 (hPD-L1) on porcine cells, which diminished human T cell- and NK cell-mediated cytotoxicity and proliferation *in vitro* [93, 94]. In a recent study, human T cells were perfused through porcine kidneys expressing hPD-L1 *ex vivo*. T cells adhered similarly to the endothelium and infiltrated the tissue of both WT and transgenic kidneys, but secretion of IFNγ, TNF, granzyme B, and perforin was decreased. The inhibition of T cell activity was confirmed *in vitro* by co-culturing renal pECs in proliferation and cytotoxicity assays [95]. The expression of inhibitory molecules proved to be quite efficient in both *in vitro* and *ex vivo* experiments. However, the confirmation of these findings *in vivo* remains to be seen.

The Fas/FasL pathway utilized by cytotoxic cells presents another potential target for inhibiting NK and CTL responses. Overexpressing membrane-bound human FasL and a human decoy Fas in pECs led to partial protection from CTL-mediated cytotoxicity [21]. However, protection from NK cell cytotoxicity was not achieved *in vitro*, in contrast NK cell migration through pECs and chemotaxis of human polymorphonuclear cells were strongly increased by the expression and cleavage of soluble FasL [107]. As an inhibitor of Fas-receptor signaling, overexpressed porcine cellular FLICE inhibitory protein (c-FLIP) in pECs resulted in a significant reduction of CTL-mediated lysis, with up to 75% suppression confirmed *in vitro* and prolonged graft survival in a rat xenotransplantation model [88].

The question of tolerance induction arises. It would be advantageous to induce tolerance of the innate and adaptive immune systems in order to prevent rejection while maintaining the ability of the immune system to protect the transplant recipient and the graft from infection. One approach to induce recipient tolerance and to avoid heavy immunosuppression could involve porcine thymus transplantation. Studies in a pig-to-humanized mouse model have demonstrated its efficacy in promoting tolerance both in mixed leukocyte reactions [113] and xenograft settings [114]. However, efficient positive selection of human T cells in the porcine thymus remains a challenge [115]. Porcine dendritic cells are likely to be pivotal cells in the initiation of CTL responses [116]. An alternative approach to achieving T-cell tolerance is therefore to use tolerogenic or regulatory dendritic cells (DC), or porcine mesenchymal stromal cells (pMSC) aiming to prevent indirect recognition [54, 117–120].

Co-Stimulation Blockade and Immunosuppressive Regimens

Costimulation blockade to inhibit xenogeneic NK and CTL responses can be achieved by two major approaches: (i) blocking receptor-ligand interactions between human or porcine APC and human T cells, using pharmacological compounds such as Abatacept/Belatacept or antibodies directed against CD40 or CD154, or (ii) by transgenic expression of genes with an inhibitory effect on costimulation in pig endothelial cells (surface expression and secretion) such as hCD152 (CTLA4 or PD-L1). Blocking of pCD86, a ligand of the activating costimulatory receptor CD28, combined with αGal downregulation, resulted in complete protection from human NK cell cytotoxicity [64]. In a previous study, Costa and colleagues demonstrated that the expression of the inhibitory costimulation receptor hCD152 (hCTLA4-Ig) on pECs resulted in the inhibition of T cell and NK cell activation [64]. The same group later developed a pig-to-mouse xenotransplantation model where they implanted pECs transduced with hCD152-hCD59 under the recipients' kidney capsules. Enhanced survival for up to 5 weeks was observed in modified xenografts compared to the control group [89]. Simultaneous blocking of NK cell activating receptors CD2 and NKG2D resulted in the suppression of NK-mediated killing *in vitro* [36]. The blockade of the $CD40^{-}CD154$ co-stimulation pathway using therapeutic monoclonal antibodies was reported to be successful in pig-to-NHP kidney transplantation [121], and in pig-to-NHP corneal transplantation, the xenografts exhibited prolonged survival (more than 933 days) [121]. This outcome was particularly attributable to the reduction in cellular infiltrates, including CTL and macrophages, as well as the diminished levels of cytokines (i.e., IFNγ, TNF, IL-2) [122]. Given that activated NK cells express CD154 (CD40L), and that binding to CD40 provides a positive signal leading to cytotoxicity [65, 123], the blockade of CD154 signaling may also be beneficial in attenuating NK cell responses. However, this hypothesis has yet to be investigated. Concurrently, the simultaneous blockade of CD28 and $CD40^{-}CD154$ pathways resulted in the complete abrogation of T cell proliferation when co-cultured with porcine APCs in a preliminary report [124]. Conversely, the cross-species interaction of CD154-pCD40 resulted in pEC activation, with the induction of E-selectin (CD62E), VCAM-1 (CD106) and SLA class-II [125].

The use of an immunosuppressive (IS) regimen is essential for prolonging xenograft survival in pig-to-NHP models despite the utilization of genetically modified porcine tissues and organs. For example, IS consisting of cyclosporin A, cyclophosphamide, and methylprednisolone delayed rejection in baboons and rhesus monkeys [68, 126]. Other experiments using hCD46 transgenic pig hearts and liver hDAF (hCD55) xenografts resulted in rejection characterized by infiltrates with $CD4^{+}$ and $CD8^{+}$ T cells [69]. Since those experiments were performed without prior T cell depletion, the effects of CTL were detrimental quite early after transplantation. T cell depletion showed benefits for renal graft survival (>400 days) in rhesus macaques [127]. Interestingly, these results were achieved by selected targeting of $CD4^{+}$ but not $CD8^{+}$ T cells, which could be explained by the failure of $CD8^{+}$ T cells to exert their function in the absence of helper T cells. Although this approach initially proves effective, $CD8^{+}$ T cells proliferate rapidly following T cell depletion therapies, with the majority comprising effector-memory T cells [128]. This was recently confirmed in a pig-to-baboon genetically modified porcine kidney and heart transplantation models [10, 129–131]. In compassionate pig-to-human cardiac xenotransplantation, IS regimen based on pig-to-baboon studies (induction with ATG and anti-CD20, anti-CD40, MMF, tacrolimus) was insufficient to provide long-term survival, presumably due to patients' comorbidities [74]. Rapamycin, an mTOR-inhibitor exhibits several properties that may confer particular benefit in

the context of xenotransplantation, including inhibition of T cell proliferation, promotion of the number of T regulatory cells, prevention of pig graft growth, and anti-inflammatory, anti-viral and anti-cancer effects [132, 133]. Whereas the use of rapamycin, a mTOR pathway blocker used as a maintenance IS treatment, has been observed *in vitro* to interfere with the xeno-specific T cell proliferative responses to pEC, in addition to modulating the pEC immunogenicity via a PD-1/PD-L1 independent pathway. Under the same conditions, belatacept only partially inhibited xenogeneic T cell proliferation and reduction of CD2 expression [134].

With regard to the impact of IS regimens on NK cells, our findings indicate that a range of NK cell functions can be suppressed by IS drugs (such as methylprednisolone and therapeutic immunoglobulins) *in vitro* [135]. Recently, clinical trials of a therapeutic CD38 antibody for antibody-mediated rejection in kidney allotransplantation have demonstrated a transient depletion of $CD16^{bright}$ NK cells and improved graft function. These results may potentially be implemented in the context of xenotransplantation [136].

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In recent years, xenotransplantation has made significant progress, especially with the advent of genetic modifications designed to attenuate hyperacute rejection. Notwithstanding these advances, acute vascular rejection and delayed xenograft rejection continue to present substantial challenges, driven by intricate interactions between the recipient's innate and adaptive immune systems and the xenograft. Genetic modifications have shown efficiency in mitigating several immune responses, yet they fall short in providing comprehensive protection from NK and CTL-mediated cytotoxicity.

In conclusion, there are multiple pivotal areas within the field of xenotransplantation research that warrant further investigation. First, additional combinations of genetic modifications to porcine tissues should be explored, such as more effective expression of human non-classical MHC class I molecules (HLA-E, HLA-G1) in addition to targeting SLA class I expression, with the aim of better suppressing NK and CTL responses. Of particular interest would also be combinations of other inhibitory pathway transgenes, such as co-expression of HLA-E, CD47, PD-L1, and so forth. Secondly, the utilization of multi-omics analyses will likely offer deeper insights into the immune response at various stages following xenotransplantation, which may ultimately result in the identification of novel therapeutic targets. Thirdly, research into local immunosuppression techniques, such as the expression of human PD-L1 or LEA29Y on xenografts, could help in minimizing systemic immunosuppression and enhancing the long-term viability of xenografts. Future work will likely also explore novel combinations of co-stimulation blockade with organs from transgenic pigs to manage T cell and NK cell activation more effectively. Allotransplantation trials will provide new insights generating strategies for addressing antibody- and cellular-mediated rejection. Another avenue for exploration is the role of glycosylation in cellular responses and the potential of inducing recipient tolerance, for example, through thymus transplantation, thereby reducing the need for lifelong immunosuppression. Ultimately, understanding and addressing the complexities of both innate and adaptive immune responses will be crucial to overcoming the remaining barriers in xenotransplantation and advancing its successful clinical application.

AUTHOR CONTRIBUTIONS

VG wrote and edited the manuscript GPY conducted several revisions of the manuscript JS conducted several revisions of the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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REVIEW
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Complement, Coagulation, and Fibrinolysis: The Role of the Endothelium and Its Glycocalyx Layer in Xenotransplantation

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In xenotransplantation, the vascular endothelium serves as the first point of contact between the recipient's blood and the transplanted donor organ. The loss of the endothelium's ability to control the plasma cascades plays a critical role in the dysregulation of the complement and coagulation systems, which greatly contribute to graft rejection and hinder long-term xenograft survival. Although it is known that an intact glycocalyx is a key feature of a resting endothelium that exhibits optimal anticoagulant and anti-inflammatory properties, the role of the endothelial glycocalyx in xenotransplantation is barely investigated so far. Here, we discuss the central role of endothelial cells and the sugar-rich endothelial glycocalyx in regulating the plasma cascades, and how the loss of these functions contributes to graft damage and rejection. We highlight the importance of preserving the regulatory functions of both endothelial cells and the glycocalyx as strategies to improve xenotransplantation outcomes.

Keywords: xenotransplantation, endothelium, endothelial glycocalyx, complement and coagulation, plasma cascades

INTRODUCTION

The luminal side of the vascular endothelium is lined by a monolayer of endothelial cells (ECs), which function far beyond merely acting as the barrier between blood and tissue. EC functions include regulating the vascular tone, mechanotransduction, permeability, oxygen and nutrient supply, vascular hemostasis, as well as immunomodulatory activities [1–6]. The outermost layer of ECs is covered by the endothelial glycocalyx, a sugar-rich layer composed of diverse sugar conjugates, such as glycoproteins, proteoglycans, and glycolipids, which serve as the first point of contact for cellular and humoral components of the immune system with the endothelium [7–10].

The well-balanced control of the plasma cascade systems – complement, coagulation, fibrinolysis, and kallikrein/kinin – by ECs is crucial in healthy and pathological conditions, including pig-to-human xenotransplantation [11]. However, this control is lost due to incompatibilities between regulatory factors provided by the porcine ECs and the human plasma components present in the recipient's blood, ultimately leading to graft damage and rejection [12–14]. The activation of the xenograft ECs and subsequent loss of the regulatory control of the plasma cascades have been shown to be the significant events leading to organ failure and rejection [15–19].

Genetic modifications have been used to overcome xenorejection due to humoral responses by eliminating the synthesis of xenoantigens [20–22]. In addition, the expression of human complement and coagulation regulatory genes in donor pigs has been used to regain control over the activation of

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the plasma cascade systems [23–26]. While impressive progress has been achieved, complement and coagulation dysregulations are still presented as key features of short- or long-term xenograft rejection in preclinical and clinical models [26–30]. This indicates that the currently available strategies to preserve the regulatory functions of xenograft ECs are not sufficient to achieve long-lasting control and graft survival.

In this review we outline the critical roles of ECs and the glycocalyx in regulating the plasma cascade systems. We also highlight the interaction of the EC glycocalyx with the recipient's plasma cascades as well as its role in xenograft rejection. Finally, we discuss the potential of EC glycocalyx protection as a possible therapeutic strategy to improve graft survival and function.

REGULATION OF PLASMA CASCADES BY ENDOTHELIAL CELLS

Normal ECs are typically in a quiescent state, displaying minimal proliferation, migration, and permeability. The surface of healthy ECs is also in an anticoagulant, anti-inflammatory, and pro-fibrinolytic state. The maintenance of this quiescent state is achieved by complex and active functions involving the continuous production and regulation of various molecules and receptors [1, 11].

Coagulation and Fibrinolysis

Coagulation and fibrinolysis are essential physiological processes that occur continuously, maintaining the delicate equilibrium between fibrin formation and breakdown in the plasma [31]. Activation of the coagulation cascade can be initiated via two pathways: the intrinsic pathway, also called the contact activation pathway, and the extrinsic pathway, which is triggered by tissue factor (TF). Both pathways result in the activation of factor X and subsequent production of thrombin, a serine protease that converts fibrinogen into fibrin, ultimately leading to clot formation. The extrinsic pathway mainly serves as a mechanism for hemostatic control and response to injury, while the activation of the intrinsic pathway has been associated with pathological clotting [32, 33].

Under normal physiological conditions, ECs prevent thrombus formation by providing a non-adhesive surface to prevent the activation of platelets and the coagulation cascade. More importantly, ECs are actively preventing thrombus formation by expressing soluble and membrane-bound molecules with anticoagulant properties [34]. ECs produce soluble molecules such as nitric oxide (NO) and prostacyclin (PGI_2) which inhibit platelet aggregation [35–37]. To inhibit coagulation via the TF pathway, ECs also express tissue factor pathway inhibitor (TFPI) [38–40]. Furthermore, as the formation of fibrin happens continuously in the plasma, ECs prevent the accumulation of fibrin by secreting tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (u-PA) [41, 42]. These two molecules will convert plasminogen to the active plasmin, which breaks down fibrin clots accumulating on the EC surface. To further enhance its anticoagulant properties, ECs bind inhibitors of coagulation factors, preventing the progression of the coagulation cascade. For instance, heparan sulfate (HS), present on the EC glycocalyx, bind the liver-derived antithrombin III (ATIII), which inhibits coagulation by preventing thrombin and other coagulation factors from binding to their substrates [43–45]. Moreover, the membrane-bound thrombomodulin (TBM) expressed on the EC surface can bind circulating thrombin and inhibit its procoagulant function, altering thrombin's affinity from binding fibrinogen to binding and activating anticoagulant protein C [34, 46, 47]. ECs also express a high affinity receptor for protein C (EPCR), which binds protein C and further enhances its TBM-thrombin complex-mediated activation [48]. The coagulation inhibitory properties of ECs are summarized in (**Table 1**).

Complement Cascade

The complement system plays a crucial role in clearing immune complexes and injured cells. It can be activated via three different activation pathways, all of which converge at the C3 level. Activation of the cascades produces opsonins (C3b, C4b), which mark the targets for subsequent removal, and the membrane attack complex (MAC, C5b-9), which directly lyses the target cells. Additionally, complement activation generates the anaphylatoxins C3a and C5a, promoting leukocyte recruitment and inflammation. However, the activation of the complement system acts as a double-edged sword because the effectors of complement activation have the potential to harm the host. To prevent this, the complement system is highly regulated through the expression of soluble and membrane-bound molecules to avoid the undesired effect of complement activation. ECs, as the first layer constantly exposed to the complement mediators in the plasma, play an indispensable role in this regulatory process.

ECs express membrane-bound components and secrete soluble molecules that prevent the activation of the complement cascade or deposition of complement activation products (**Table 2**). ECs express the surface molecules CD46 and CD55, which inhibit the activation of complement pathways, and CD59, which prevents the formation of MAC on the surface of ECs [50, 51, 54]. ECs also secrete C1 inhibitor (C1-INH), clusterin, factor H, and factor I, which prevent the formation of complement effectors at various stages [49, 52, 53, 55, 56].

Both the complement and coagulation systems comprise of serine proteases with common ancestral genes [57, 58]. There is substantial evidence of crosstalk between complement and coagulation factors, leading to mutual engagement of both systems. For instance, proteases such as thrombin, factor XII (Hageman factor), and plasmin play roles in both coagulation and complement activation [59–62]. This crosstalk also influences the regulation of the plasma cascade pathways by ECs through shared complement-coagulation regulators such as C1-INH, TBM, TFPI, CD46, CD55, and CD59 [58, 63–65]. Consequently, a lack of function of the previously mentioned inhibitors, can also contribute to concurrent activation and amplification of complement and coagulation pathways under pathological conditions, resulting in excessive activation of both systems [64, 66, 67].

TABLE 1 | Anticoagulant properties of endothelial cells.

Molecule	Form	Target action	Function	Ref
Nitric Oxide (NO)	Soluble gas	Platelets	Diffuses to nearby platelets and prevents calcium release, which ultimately prevents platelet activation and aggregation	[36, 37]
Prostacyclin (PGI_2)	Soluble protein	Platelets	Inhibition of platelet activation through calcium release inhibition	[35]
Heparane Sulfate (HS)	Membrane-bound glycosami noglycans	ATIII	Bound ATIII inhibits thrombin (factor II) and other serine proteases binding to their substrates	[43, 44]
Tissue Factor Pathway Inhibitor (TFPI)	Soluble protein	Tissue factor (Factor III)	Limits the TF (factor III) activity which initiates prothrombin transformation into thrombin	[38, 39]
Thrombo modulin (TBM)	Membrane-bound protein	Thrombin, protein C	Binds to thrombin and reduces the ability of thrombin to convert fibrinogen into fibrin TBM-thrombin complex enhances protein C activity to inhibit coagulation factors Va and VIIIa	[46, 48]
t-PA	Soluble protein	Plasminogen	Activates plasminogen into plasmin and initiates fibrinolysis	[31, 41]
u-PA	Soluble protein	Plasminogen	Activates plasminogen into plasmin and initiates fibrinolysis	[31, 41]
EPCR	Membrane-bound protein	Protein C	Binds and promotes protein C activation by presenting protein C to TBM-thrombin complex, ultimately inhibiting factor Va and VIIIa	[48]

TABLE 2 | Complement regulatory properties of endothelial cells.

Molecule	Form	Target action	Function	Ref
C1 inhibitor	Soluble protein	C1	Inhibits the activation of complement C1	[49]
CD46 (MCP)	Membrane-bound protein	C3, C4	Mediates the cleavage of C3b and C4b	[50]
CD55 (DAF)	Membrane-bound protein	C3 convertase C5 convertase	Destabilizes C3/C5 convertases in the classical and the alternative pathways	[51]
Factor H	Soluble protein	C3b	Inactivates the C3 and C5 convertases	[52]
Factor I	Soluble protein	C3b, C4b	Cofactor for the C3b and C4b cleavage by factor H	[53]
CD59	Membrane-bound protein	C5b-9	Prevents C5b-9 formation	[54]
Clusterin	Soluble protein	C5b-9	Prevents C5b-9 formation	[55, 56]

Genetic Modification Strategies to Overcome Complement and Coagulation Dysregulation in Xenotransplantation

In xenotransplantation, interaction of the porcine ECs with the human plasma cascades presents great immunological challenges due to the presence of xenoantigens on porcine ECs, and molecular incompatibilities between porcine plasma cascade regulators and human plasma cascade components [21, 22, 68]. The porcine glycocalyx presents sugars carrying α-Gal, N-glycolylneuraminic acid (Neu5Gc), and Sd^a epitopes, which can be recognized as foreign by natural antibodies circulating in human blood, and have been shown to play a significant role in the immune response to pig grafts in xenotransplantation [22, 68–71]. Antibody-mediated complement activation will lead to hyperacute rejection, a rapid destruction of the xenograft due to the complement attack, accompanied by interstitial hemorrhage, edema, and microvascular thrombosis [21, 72, 73]. The lack of complement and coagulation protection on the surface of ECs due to the interspecies molecular incompatibilities also leads to complement-mediated injury and coagulopathy, which are evident in various stages of xenograft rejection [74–76].

The first approach to avoid activation of complement on the EC surface in xenotransplantation is the deletion of porcine xenoantigens, which was shown to prevent hyperacute rejection [20, 24, 70, 77]. Another approach to provide better control of the plasma cascades by the endothelium is the transgenic overexpression of human complement and coagulation regulatory factors [16, 23–26]. Strategies to overcome the complement and coagulation dysregulation are illustrated in **Figure 1**. Genetically modified pigs carrying single or multiple xenoantigens knockouts and expressing varying combination of plasma cascade regulatory proteins have been developed and show different survival rates [26, 81, 87–89].

THE ROLE OF THE ENDOTHELIAL GLYCOCALYX IN PLASMA CASCADE REGULATION

The function of ECs, from mechanotransduction, maintenance of vascular integrity and vascular tone, as well as regulating the interaction of EC with the immune components of the blood, is profoundly influenced by their glycocalyx component [90]. While the term glycocalyx broadly refers to surface sugar layer of any cells, the EC glycocalyx specifically refers to the carbohydrate-rich layer on the luminal side of ECs. The thickness and the diversity of the EC glycocalyx is influenced by the shear stress, plasma proteins, and extrinsic glycosylation factors in the blood [3, 10, 91–93]. The EC glycocalyx is made up of diverse sugar conjugates, including proteoglycans, glycoproteins, and glycolipids, facilitating the interaction of ECs with various immune mediators of the plasma cascades.

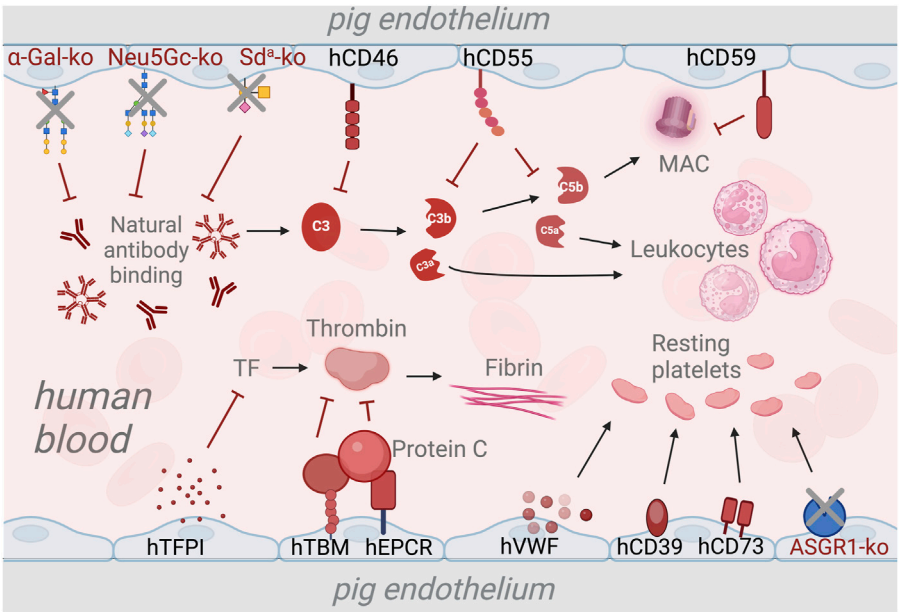


FIGURE 1 | Strategies to overcome the complement and coagulation dysregulation in xenotransplantation using genetic modifications. The deletion of genes responsible for xenoantigen epitopes prevents the binding of natural antibodies and subsequent activation of the complement system [20, 21, 24]. Transgenic expression of human complement regulatory genes such as human CD46 (hCD46), hCD55, and hCD59 inhibits the activation of the complement cascade and the formation of membrane attack complex (MAC) [75, 78, 79]. The expression of human tissue pathway inhibitor (hTFPI) is used to prevent activation of the coagulation cascade via the tissue factor (TF) pathway [80]. Additionally, Human thrombomodulin (hTBM) and endothelial protein C-receptor (hEPCR) are expressed to inhibit thrombin activation, thus preventing fibrin formation [26, 81]. To maintain the platelets in a resting state, transgenic expression of human von Willenbrand Factor (hVWF), triphosphate diphosphohydrolase-1 (human ENTPDase-1 or hCD39) and human CD73 [82–84]. Deletion of the asialoglycoprotein receptor-1 (ASGR1) gene is used as a strategy to reduce human platelet destruction by pig livers [85, 86].

Proteoglycans are glycoproteins comprising a core protein (e.g., syndecan, glypican, and perlecan) that carries covalently attached linear polysaccharide glycosaminoglycans (GAGs). The main components of the EC glycocalyx GAGs are HS, chondroitin sulfate (CS) and dermatan sulfate (DS) [10, 94]. The EC glycocalyx also contains the non-sulfated GAG hyaluronan (HA), typically anchored by the CD44 receptor, or as a soluble component [95, 96]. GAGs are the most abundant sugars in the EC glycocalyx, contributing significantly to its molecular mass and negative charge, creating a transendothelial gradient for fluid transit, masking the adhesion molecules, and capturing circulating plasma proteins [97, 98]. GAGs are also essential in maintaining the quiescent state of ECs. They act as mechanotransducers, signaling to maintain EC function via the production of NO [90, 99]. GAGs can bind and modulate the activity of several growth factors, antiangiogenic, and inflammatory molecules such as VEGF, FGF, IL8, MMPs, angiopoietin-1, thrombospondin-1, endostatin, and PAI-1 [100–105]. Additionally, GAGs have binding sites for xantine-oxydoreductase (XOD) and endothelial superoxide dismutase (eSOD), preventing oxidative damage to ECs [106, 107].

HS is one of the most extensively studied GAGs and builds up most of the sugar components of the EC glycocalyx. HS is known for its critical role in various biological functions, from serving as a binding domain for signal transduction molecules to regulating the plasma cascade and immune interactions [108]. Plasma proteins bind to HS via their heparin-binding domain. This binding is specific, and through the differential expression of HS, ECs can selectively determine which plasma proteins will bind to the surface [109, 110]. HS on the EC glycocalyx can bind and potentiate the activity of complement and coagulation regulatory factors, including factor H, C1-INH, and ATIII, providing an anti-inflammatory and anticoagulant surface [44, 111–115]. Binding of growth factors and cytokines such as VEGF, FGF, and IL8 is predominantly facilitated by HS [43, 103, 116, 117]. HS also serves as a binding site for P-selectin and L-selectin, potentially acting as a decoy receptor to influence cell adhesion dynamics [118, 119]. Moreover, HS is also involved in the regulation of the kinin-kallikrein system through its capacity to recruit kinin precursors and mediate the activation of high molecular weight kininogen [120, 121].

The terminal glycans of mammalian glycoproteins and glycolipids are typically substituted by the negatively charged sialic acid, which plays an essential role in physiological and pathological processes, including complement, coagulation, and inflammation [72, 122–124]. Many important receptors and regulatory proteins on the EC surface carry N- and O-linked oligosaccharide chains and are classified as glycoproteins [94, 125]. The interaction between ECs and leukocytes is controlled by adhesion molecules such as siglecs and selectin receptors, that are heavily influenced by the cellular glycosylation patterns of these

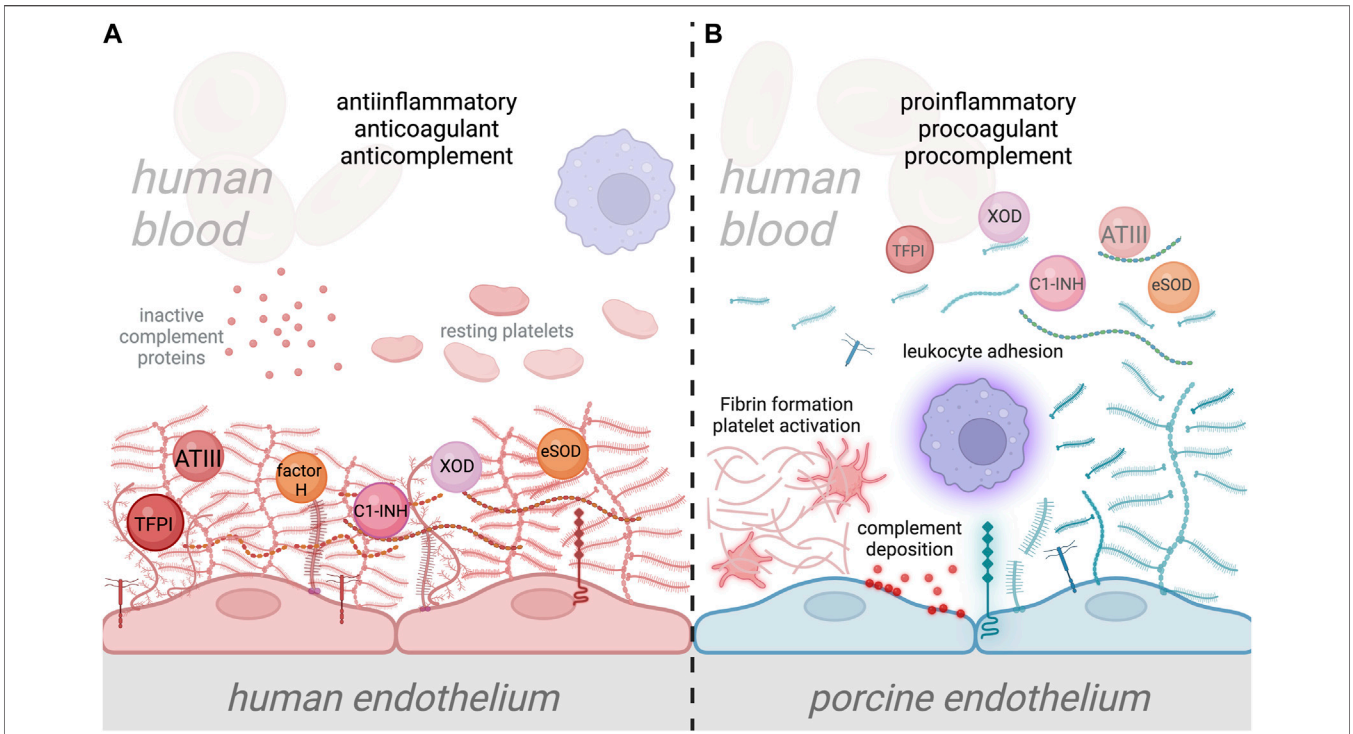


FIGURE 2 | Interaction of human plasma cascade proteins with human and porcine glycocalyx. **(A)** Normal interaction between human plasma proteins and the human endothelial cell (EC) glycocalyx, where plasma cascade regulatory molecules such as antithrombin III (ATIII), tissue factor pathway inhibitor (TFPI), Factor H, and C1-inhibitor (C1-INH) are specifically bound to EC glycocalyx, providing anticomplement, anticoagulant, and anti-inflammatory properties to the ECs [44, 112–114]. The human EC glycocalyx also binds antioxidant molecules such as XOD and SOD, thereby preventing oxidative damage to ECs [106, 107]. Attachment of leukocytes is prevented by the negatively charged EC glycocalyx and the shielding of leukocyte binding receptors [138, 157]. **(B)** In xenotransplantation, the disparity between human and porcine EC glycocalyx composition, which likely alters its structure and function, could lead to inefficient binding of protective plasma proteins. This could result in the transition of the endothelium from a quiescent into an activated state, which leads to glycocalyx shedding. Disruption of the porcine EC glycocalyx and loss of its anticoagulant properties will result in activation of the coagulation system, platelet recruitment and fibrin formation. Loss of anticomplement properties will also lead to the deposition of complement proteins on the EC surface, along with the amplification of inflammation. Additionally, shedding of the porcine EC glycocalyx will increase leukocyte attachment to the surface of EC due to the loss of leukocyte binding receptor shielding. Ultimately, this condition could lead to xenograft damage and failure.

proteins [126, 127]. Factor H can bind to sialic acid, providing a protective layer on ECs and shielding the cells from the deposition of complement [128–131]. Glycosphingolipids (GSLs), a diverse group of glycolipids containing one or more glycans anchored to a ceramide, are known to play important roles in many cellular processes, such as signal transduction, cell-cell interactions, and immune response regulation, including the complement and coagulation systems [132–135]. While the role of GSLs has been extensively studied in neurodegenerative diseases, their role in the context of xenotransplantation immunology is less well-understood.

Glycocalyx Shedding

Due to its pivotal role in maintaining the normal function of ECs, destruction of the EC glycocalyx will lead to impairment of EC function and overall vascular hemostasis. Glycocalyx shedding is observed when ECs are activated and exhibit a pro-inflammatory state, including in trauma-related injury, ischemia/reperfusion injury, hemorrhagic shock, and hyperglycemia [10, 136–139]. In xenotransplantation, glycocalyx shedding associated with complement and coagulation activation after xenogeneic treatment has been observed *in vitro* and *in vivo* [140–144]. Disruption of the EC glycocalyx may hamper its mechanotransduction ability and induce edema due to elevated vascular permeability [145–149]. Shedding of the EC glycocalyx will result in the loss of anticoagulant and anti-inflammatory properties of the EC, leading to increased coagulation, decreased pro-fibrinolytic activity, increased complement deposition, and adhesion of leukocytes to the EC surface [138–140, 150].

Shedding of the EC glycocalyx during inflammation can be mediated by various glycocalyx modifying factors such as reactive oxygen species (ROS), matrix metalloproteinases (MMPs), and glycan-degrading enzymes (heparanase, hyaluronase, sulfatase, sialidase, etc.) [9, 10, 139, 151]. Other proteins with enzymatic functions such as thrombin, elastase, proteinase 3, plasminogen, as well as cathepsin B also contribute to degradation of the glycocalyx [152–154]. In cardiovascular diseases associated with vascular inflammation, the observed glycocalyx shedding can be mediated by multiple glycocalyx-degrading mediators [139, 155, 156].

Interaction of the Porcine Glycocalyx With Human Plasma Proteins in Xenotransplantation

One significant concern, and barely studied so far, are the possible differences in the binding of human plasma proteins to the human and porcine glycocalyx (**Figure 2**). The absence of glycans containing α-Gal, Neu5Gc, and Sd^a epitopes in humans highlights the species-specific differences in sugar synthesis and expression, as well as the disparity in glycocalyx composition between humans and pigs [21]. Due to its different composition, the porcine glycocalyx may have varying structure and affinities for human plasma cascade regulators such as ATIII, factor H, and C1-INH, which could reduce the anti-inflammatory and anticoagulant properties of the xenografted EC surface once a porcine organ has been grafted into a human recipient. Discrepancies in the binding affinities to human growth factors, cytokines, and chemokines could likely also cause a systemic effect due to loss of control of dissociated and active molecules by ECs [103, 109, 158]. Similarly, possible differences in enzyme kinetics and affinities between the porcine glycocalyx and glycocalyx-modifying enzymes could also affect the glycocalyx dynamics and function. Therefore, despite the effort to substitute the interspecies complement and coagulation incompatibility on a protein level in xenotransplantation, EC function in controlling the plasma cascade regulation might still not be optimal without proper functioning of the glycocalyx [144].

Initially, the removal of sugar xenoantigens from xenograft donor pigs by genetic engineering was implemented to overcome the antibody-mediated rejection and has been proven effective [20, 24, 70, 77]. However, these genetic modifications have been found to influence the expression of other sugars and may even lead to the synthesis of novel glycans, which is likely to influence the structure, composition, and immunological function of the porcine EC glycocalyx [159, 160]. Therefore, further studies are necessary to determine how these alterations in sugar composition and the emergence of neoglycans influence the phenotype and physiological function of the porcine EC glycocalyx.

EXPLORING EC GLYCOCALYX PROTECTION IN XENOTRANSPLANTATION

Protecting ECs and their glycocalyx is critical for improving graft survival and function, highlighting the importance of targeted therapy for preserving and maintaining the quiescent state of the endothelium. Current strategies, including genetic engineering, might indirectly protect the glycocalyx to a certain extent by preventing antibody binding, complement activation, and controlling the coagulation system. Additionally, anti-inflammatory and anticoagulant agents, such as corticosteroids and heparin, which are already incorporated in current immunosuppressive regimens in xenotransplantation, have been shown to prevent the degradation of the glycocalyx [161–163]. However, although evidence specific to xenotransplantation remains limited, shedding of the glycocalyx continues to be a significant issue in pathological conditions related to vascular inflammation. Shedding of the glycocalyx in a setting of (xeno)transplantation occurs immediately after graft reperfusion (ischemia reperfusion injury), related to trauma and inflammation, or long-term related to chronic rejection [136–138, 156, 164, 165]. Therefore, strategies to prevent shedding of the glycocalyx, or to functionally replace glycocalyx components, might be needed in clinical xenotransplantation.

Generally, glycocalyx protection strategies employ therapeutic agents that interact with one or multiple glycocalyx-modifying factors, including inhibiting the glycocalyx-degrading enzymes, oxidative stress, and inflammation. Molecules such as angiopoietin, hydrocortisone, ATIII, berberine, and S1P prevent the degradation of glycocalyx through the inhibition of MMPs [10, 153, 166, 167]. SOD acts as antioxidant and helps to mitigate oxidative stress associated with ischemia-reperfusion injury, thus protecting the glycocalyx [149]. Sulodexide can protect the glycocalyx by inhibiting heparanase-1 [168]. While those therapies have been used in other vascular disorders, the protective effects on porcine xenografts need further evaluation, especially since existing studies do not fully address the structure, function, and dynamics of the glycocalyx in a xenotransplantation context.

Glycan-specific approaches have also been employed for glycocalyx protection and restoration. Emerging therapies such as bioengineering approaches also offer innovative strategies, for instance, by engineering the EC to rebuild the glycocalyx, which has been shown to minimize graft injury and rejection [169]. Other strategies use a class of molecules which can be termed "endothelial cell protectants." One such molecule, low molecular weight dextran sulfate of 5000 MW (DXS), was shown to be protective both *in vitro* and in a small animal model of xenotransplantation [170, 171]. DXS has also been shown to prevent ischemia/reperfusion injury in large animal models [172, 173]. Similarly, multimeric sulfated tyrosine has been shown to help maintain and restore the glycocalyx layer [174]. While currently there are no clinically approved drugs which act as EC protectants, this class of substances has proved to be successful in preclinical experiments and, in our view, by keeping the endothelium in a quiescent state, has great potential as an additional drug treatment in xenotransplantation.

CONCLUSION

The interaction between the porcine glycocalyx and the human plasma cascades involves complex, yet poorly understood dynamics that are critical to the success of xenotransplantation. To address these challenges, a deeper understanding of interspecies glycocalyx differences and their impact on immune responses, coagulation, and EC function is required. New insights into the structure and function of the porcine and human glycocalyx, as well as the mechanisms of glycocalyx degradation and regeneration

in xenotransplantation, hold the potential to unlock novel approaches to preserve the endothelial glycocalyx. Protective strategies for the glycocalyx, combined with the already available strategies to prevent complement and coagulation dysregulation, can be explored as an approach to improving the outcomes of xenotransplantation.

BIOGRAPHY

Robert Rieben was born in Bern, Switzerland, where he also studied biology. During his PhD he worked on blood group ABO antibodies and, via contacts with Rafael Oriol and David Cooper, got in touch with xenotransplantation. As a postdoc in Mohammed Daha's lab in Leiden/NL, he started to work on the complement system and endothelial cells. At the same time, he collaborated in the first EU-funded xenotransplantation research projects "Glycoimmunology", led by David Joziasse, and "Xenotransplantation", led by Bo Samuelsson. Back in Switzerland, he started his own research group and continued to work on the concept of endothelial cell protection in xenotransplantation, allotransplantation, and ischemia/reperfusion injury. For the past 12 years, Robert Rieben has been affiliated to the German Transregio 127 research project on xenotransplantation, led by Bruno Reichart and Eckhard Wolf, and since 2020 he leads a Sinergia project of the Swiss National Science Foundation together with Joerg Seebach and Eckhard Wolf.

AUTHOR CONTRIBUTIONS

MG: writing (original draft, review, editing). RR: writing (review, editing), supervision. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Progress in Orthotopic Pig Heart Transplantation in Nonhuman Primates

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Xenotransplantation of porcine hearts has become a promising alternative to human allotransplantation, where organ demand still greatly surpasses organ availability. Before entering the clinic, however, feasibility of cardiac xenotransplantation needs to be proven, ideally in the life supporting orthotopic pig-to-nonhuman primate xenotransplantation model. In this review, we shortly outline the last three decades of research and then discuss in detail its most recent advances. These include the genetic modifications of donor pigs to overcome hyperacute rejection and coagulation dysregulation, new organ preservation methods to prevent perioperative xenograft dysfunction, experimental immunosuppressive and immunomodulatory therapies to inhibit the adaptive immune system and systemic inflammation in the recipient, growth control concepts to avoid detrimental overgrowth of the porcine hearts in nonhuman primates, and lastly, the avoidance of porcine cytomegalovirus infections in donor pigs. With these strategies, consistent survival of 6–9 months was achieved in the orthotopic xenotransplantation model, thereby fulfilling the prerequisites for the initiation of a clinical trial.

Keywords: orthotopic heart transplantation, xenotransplantation, organ perfusion, costimulation blockade, genetically-modified pig

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INTRODUCTION

Since the first orthotopic transplantation of a porcine heart into a baboon 30 years ago [1], there has been tremendous progress towards the ultimate goal - moving cardiac xenotransplantation (xHTx) into the clinic. In the beginning, most research focused on the non-life supporting heterotopic abdominal xHTx model [2–11], primarily to study immunosuppressive regimes to overcome hyperacute and acute rejection (reviewed in detail [12–14]). While xenograft survival in this model had steadily increased from several weeks to months and years, results in the life supporting orthotopic xHTx model were not encouraging: until 2017, most experiments lasted only a few days and many recipient animals were lost during the first 48 h [1, 2, 15–21]; maximum survival was 57 days [21]. These results were far from the recommendations devised by an expert committee of the International Society for Heart and Lung Transplantation (ISHLT) in 2000 [22]: as a prerequisite for a clinical application of xHTx, consistent 90-day survival of 60% of the animals in a life supporting xHTx model was deemed necessary. In the following 5 years, research in the field yielded many new insights, leading not only to improvements in both survival consistency and survival time [23–27], but also to the first clinical xenotransplantation of a porcine heart into a human [28].

TABLE 1 | Orthotopic pig-to-baboon cardiac xenotransplantation experiments in the last three decades (1994–2023).

Year	Author	Donor genetics	Organ preservation	Immunosuppression	Immunomodulation	Growth inhibition	Survival
1994	Fukushima Nz [1]	WT	static	CsA, DSG	Immunadsorption		3 days, 4 days, 4 days, 5 days, 6 days, 6 days, 8 days, and 16 days
1998	Xu H [15]	WT	static	CsA, MTX, Cs, ATG	TLI		3 h, 18 days, 19 days
1998	Schmoeckel M [16]	hCD55	static	CsA, CyP, Cs			6 h, 6 h, 9 h, 10 h, and 18 h, 4 days, 5 days, 5 days, 5 days, and 9 days
1998	Waterworth PD [2]	hCD55	static	CsA, CyP, Cs			0 h, 0 h, 5 days, 5 days, 9 days
2000	Vial CM [17]	hCD55	static	CsA, CyP, Cs, MMF			39 days
2005	Brandl U [18]	hCD55	static	CyP, Cs, Tac, mTOR-I, ATG	aGAL polymer[a]		1 day, 30 h, 9 days, 25 days
2005	Brenner P [19]	hCD55	static	CsA, CyP, Cs, MMF			1 h, 11.1 days, 13.1 days, 20 days
2007	Brandl U [20]	hCD55[a], hCD46[a]	static	CyP, Cs, Tac, mTOR-I, ATG, Rix[a]	aGAL polymer[a]		1 day, 9 days; 0 h, 30 h; 0 h, 20 h, 14 days, 25 days; 5.5 h, 9.5 h, 34 h, 3 days, 4 days
2008	McGregor CGA [21]	hCD46	static	Tac, mTor-I, ATG	aGAL polymer		34 days, 40 days, 57 days
2018	Längin M [23]	GGTA1-KO, hCD46, hTBM	static[a], perfusion[a]	Cs, MMF, anti-CD40 ab[a]/ anti-CD40L PAS Fab[a], ATG, Rix	C1-I, anti-IL1R, anti-IL6R, anti-TNFa	mTOR-I[a], ACE-I[a], BB[a]	1 day, 1 day, 1 day, 3 days, 30 days; 4 days, 18 days, 27 days, 40 days; 51 days, 90 days, 90 days, 182 days, 195 days
2020	DiChiacchio L [24]	GGTA1-KO, CMAH-KO[a], B4GALNT2-KO[a], hCD55[a], hCD46, hTBM[a], hEPCR[a], hCD47[a], hHMOX1[a], hvWF[a]	static	Cs, MMF, anti-CD40 ab, ATG, Rix	CVF		2 h, 7 h, 18 h, 22 h, 26 h, 40 h
2020	Reichart B [25]	GGTA1-KO, hCD46, hTBM	perfusion	Cs, MMF, anti-CD40 ab, ATG, Rix	C1-I, anti-IL1R, anti-IL6R, anti-TNFa	mTOR-I, ACE-I, BB	15 days, 27 days, 90 days, 90 days
2022	Mohiuddin MM [26]	GGTA1-KO, CMAH-KO[a], B4GALNT2-KO[a],hCD55[a], hCD46[a], hTBM[a], hEPCR[a],hCD47[a],hHMOX1[a], hvWF[a]	static[a] (blood cardio), perfusion[a]	Cs, MMF, anti-CD40 ab, ATG, Rix	CVF[a], C1-I[a], anti-IL6R, anti-TNFa	GHR-KO[a]	6 h, 4 days, 29 days, 57 days; 12 h, 6 days, 8 days; 84 days, 95 days; 182 days, 264 days
2022	Cleveland DC [27]	GGTA1-KO, hCD55[a], hCD46[a], hTBM[a]	static (del Nido[a])	Cs, mTOR-I, anti-CD40 ab, ATG, Rix	C1-I, anti-TNFa	BB[a]	3 h; 4 h, 90 days, 241 days

WT, wildtype; CsA, cyclosporine A; DSG, desoxyspergualin; MTX, methotrexate; Cs, corticosteroids; ATG, anti-thymocyte globulin; CyP, cyclophosphamide; MMF, mycophenolate mofetil; Tac, tacrolimus; mTOR-I, mTOR, inhibitor (sirolimus, temsirolimus); Rix, rituximab; anti-CD40 ab, anti-CD40 monoclonal antibody; anti-CD40L PAS Fab, PASylated anti-CD40L antibody fragment; TLI, total lymphoid irradiation; C1-I, C1-esterase inhibitor; anti-IL1R, interleukin 1 receptor blocker; anti-IL6R, interleukin 6 receptor blocker, anti-TNFα, tumor necrosis factor alpha inhibitor; ACE-I, angiotensin-converting enzyme inhibitor; BB, beta blockers; GHR-KO, growth hormone receptor knockout.

[a]*drug/genetic modification used only in some experiments of the respective study.*

In the following review, we specifically aim at a detailed overview on this recent period and highlight key findings that facilitated the progress of xHTx.

FIRST DECADE (1994–2003)

In the first decade of orthotopic xHTx, research in Loma Linda (CA, USA) and Cambridge (UK) focused on overcoming hyperacute and acute rejections. Survival rates where however short and inconsistent (**Table 1**; **Figure 1**) [1, 2, 15–17], with an overall median survival of 6.6 ± 1.6 (SEM) days and a maximum survival of 39 days [17]. At that time, the main cause for hyperacute rejection after porcine xHTX into non-human primates – the binding of preformed antibodies to the carbohydrate antigen galactose-α1,3-galactose (αGAL) on porcine endothelial cells – could not be avoided since cloning techniques to knock-out αGAL were not established in the pig

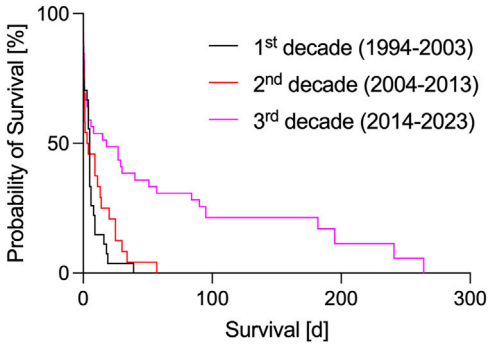


FIGURE 1 | Survival after orthotopic pig-to-baboon cardiac xenotransplantations from 1994 to 2023. Data taken from [1, 2, 15–21, 23–27].

[29]. By adding the human transgene *hDAF* (or *hCD55*) to the donor pig genome [30], complement activation following binding of anti-pig-antibodies to αGal epitopes could be attenuated, and graft survival was prolonged [2, 16, 17]. However, without further genetic modifications and the "standard" clinical immunosuppression of that time (i.a. cyclosporin A, methotrexate, cyclophosphamide, corticosteroids), expectations of a fast translation into the clinic could not be met.

SECOND DECADE (2004–2013)

With the discovery of the αGAL epitope (reviewed in [31]), research groups in Rochester (MN, United States) and Munich (Germany) began infusing synthetic GAL oligosaccharides in recipients of orthotopic xHTx, with the aim of binding and inactivating anti-αGAL-antibodies, and finally preventing hyperacute rejection reactions [18, 20, 21]. As another strategy, immunoadsorption was explored [32–34]. The human complement-regulating transgene *hCD46* was introduced to counteract complement activation independent of antibody binding [35] and tested in orthotopic xHTx experiments [18, 21]. New clinically available immunosuppressants, such as mycophenolate mofetil, tacrolimus or rapamycin, were used, with and without B cell and T cell depletion (anti-thymocyte globulin (ATG), rituximab) for induction [18–21]. With these improvements, graft rejection could be delayed, and overall mean survival increased to 10.9 ± 2.9 (SEM) days (**Table 1**; **Figure 1**), with the longest single survivor reaching 57 days [21].

Although these results were promising, they still by far did not meet the prerequisites for a clinical study as defined by the ISHLT [22]. Especially puzzling was the fact, that the results after heterotopic abdominal pig-to-baboon xHTx experiments, with survivals of up to 179 days [5], were superior to those after orthotopic xHTx. The main culprit was "Perioperative Cardiac Xenograft Dysfunction" [36] (PCXD), a systolic organ failure that led to graft loss in up to 60% within the first 48 h after orthotopic transplantations, without any signs of rejection [35]. Possible explanations for PCXD included various xeno-specific factors, such as the use of young donor organs, inflammatory effects of preformed non-GAL antibodies, incompatibility between porcine and primate plasma; donor-specific factors, notably the sensitivity of the porcine heart to cardiopulmonary bypass and ischemia/reperfusion injury, were also discussed [reviewed in [36]].

As successful translation to the orthotopic pig-to-baboon model seemed more and more impossible, researchers focused on refining the heterotopic abdominal model; the question was raised, if further orthotopic xHTx experiments were at all needed for clinical application [37].

THIRD DECADE (2014–2023)

With the creation of knockout pigs lacking the gene coding for αGAL (*GGTA1*-KO) [38] and the addition of human thrombomodulin (*hTBM*) to overcome thrombotic microangiopathy due to interspecies coagulation incompatibilities [39], survival rates after heterotopic abdominal xHTx increased even further: In 2016, M. Mohiuddin reached a median graft survival of 298 days in five baboons (maximum survival 945 days) with triple genetically modified donor piglets (*GGTA1-KO*, *hCD46*, and *hTBM*) and an immunosuppression based on an experimental chimeric CD40/CD40L costimulation blockade (mouse/rhesus monkey clone 2C10R4) [11]. In two of these long-term experiments, graft rejection was intentionally triggered by discontinuation of the costimulation blockade, demonstrating the importance of this specific therapy.

By translating the knowledge gained from these heterotopic abdominal xHTx experiments and new approaches in organ preservation and growth inhibition, the field of orthotopic pig-to-baboon xHTx was stimulated by the work of the Munich group [23] and, subsequently, further advanced by the group of M. Mohiuddin in Baltimore (MD, United States) [26]. Overall mean survival in this decade increased to 51.8 ± 11.5 (SEM) days (**Table 1**; **Figure 1**), with the longest single survivor reaching 264 days [26]. And even more so, in 2020 the recommendations of the ISHLT [22] were finally fulfilled for the first time [25], paving the way for a first clinical trial. The most important advances of this last decade are now reviewed in more detail in the following.

GENETIC MODIFICATIONS

The longest surviving grafts in recent orthotopic pig-to-baboon xHTx studies included at least the following three genetic modifications [23, 26, 27]: *GGTA1-KO* for the removal of the αGAL epitope, insertion of the complement regulator *hCD46*, and *hTBM* to overcome interspecies coagulation incompatibilities. With these minimum set of modifications, maximum survival times were 195 [23], 241 [27], and 264 days [26]. In the latter experiment, however, the porcine donor also carried several additional modifications: KOs of β-1,4-N-acetyl-galactosaminyl transferase 2 (*B4GALNT2-KO*), and growth hormone receptor

(*GHR-KO*); additional transgenes included a second complement regulator protein (*hCD55*), the human endothelial protein C receptor (*hEPCR*) and the human signal regulatory protein alpha *hCD47*. It remains unclear to what extent these additional modifications contributed to graft survival.

For human recipients, KOs of the genes *B4GALNT2* and cytidine monophosphate-N-acetylneuraminic acid hydroxylase (*CMAH)* in addition to *GGTA1-KO* are important for xHTx because of preformed antibodies against their respective gene products N-glycolylneuraminic acid (Neu5Gc) and a glycan corresponding to the human Sd(a) blood group antigen (β4GAL) ("triple KO-pig," reviewed in [40]). In the nonhuman primate (NHP) model, by contrast, *CMAH*-KO has been reported to be disadvantageous due to the presumed exposure of another, yet unknown xenoantigen [41, 42]. This is supported by results from Mohiuddin's study, in which maximum survival of grafts with *CMAH*-KO was only 8 days [26].

In the last few years, a plethora of genetical modifications have been described, including deletion of genes coding for carbohydrate antigens and expression of complement and coagulation regulatory, as well as immunomodulatory proteins (reviewed in detail elsewhere [40]). Further studies are needed to better define which combination of additional modifications might prove beneficial for graft survival. Based on existing data [23, 26, 27], at least a minimum of one complement regulator and one coagulation regulatory protein in addition to *GGTA1-KO* seems to be required.

ORGAN PRESERVATION

In the previous two decades, clinically applied cold crystalloid cardioplegic solutions were used to preserve the porcine donor hearts. Byrne and McGregor assumed that an increased sensitivity of the porcine heart to ischemia/reperfusion injury was a major contributor to PCXD [36]. Although PCXD was overcome in the heterotopic thoracic xHTx model [43] and in singular cases of orthotopic xHTx experiments [21], consistent survival could not be achieved using this conservation technique. In 2016, Steen et al. developed a cold, non-ischemic preservation method with an oxygenated, hyperoncotic, erythrocyte containing cardioplegic solution, with which pig hearts were successfully preserved for up to 24 h [44]. The Munich group adopted this preservation method for orthotopic xHTx: after cardiopulmonary bypass had stopped, cardiac function was preserved, and inotropic support was reduced to a minimum [23, 44]. Several explanations have been postulated to be responsible for the results after non-ischemic porcine heart preservation: addition of oxygenated erythrocytes to minimize myocardial ischemia/reperfusion injury, hypothermia to reduce metabolic needs, continuous delivery of nutrients and removal of toxic metabolites, high oncotic pressure of the preservation medium and strict pressure-/flow-controlled coronary perfusion to inhibit edema formation and reduce capillary damage, and lastly, physiological levels of catecholamines, cortisol and thyroid hormones for maintenance of myocardial energy stores [45–48].

After several discouraging experiments with static ischemic preservation [24], Mohiuddin's group also adopted the non-ischemic preservation technique [26]. Additionally, ischemic preservation with blood cardioplegia/Del Nido solution yielded promising results [27, 49], especially when the ischemic periods were kept very short [50].

IMMUNOSUPPRESSIVE THERAPIES

The addition of CD40/CD40L costimulation blockade to an immunosuppression based on MMF, steroids and preoperative T and B cell depletion (ATG, rituximab) was an important step for prolonging xenograft survival after heterotopic abdominal and orthotopic pig-to-NHP xHTx [11, 23]. The first study showing that CD40/CD40L costimulation blockade was superior to conventional immunosuppression was done by Bühler et al [51] in 2000, and it was first used in xHTx by Kuwaki et al. in 2005 [5]. Since then, several experimental antibodies have been tested in preclinical xenotransplantation studies (reviewed in detail in [52]). Whereas initial results in heterotopic xHTx with anti-CD40L antibodies (clone 5C8H1) were very promising [53], first-generation antibodies proved to be thrombogenic in clinical trials due to activation of thrombocytes via binding to the FcγRII receptor [54] and were subsequently abandoned. Most recent results in preclinical orthotopic cardiac xenotransplantation were achieved with the chimeric anti-CD40 antibody clone 2C10R4 [25, 26]. For clinical use, a humanized version of this antibody has been developed by Kiniksa Pharmaceuticals (KPL-404, abiprubart), which completed a phase I trial [55] and recently commenced a phase II clinical trial for treatment of Sjögrens disease; this antibody was also applied in the first clinical xenotransplantation of a porcine heart into a human [28]. Several other pharmaceutical companies have antiCD40/CD40L antibodies in their pipelines (overview in [56, 57]). Until now, however, none of these antibodies have been approved for clinical use.

IMMUNOMODULATORY THERAPIES

After pig-to-NHP xenotransplantation, systemic inflammation reactions were observed - termed "Systemic Inflammation in Xenograft Recipients" (SIXR) -, defined by an increase in inflammatory markers (C-reactive protein, histones, serum amyloid A, D-dimer, cytokines, chemokines) and a decrease in free triiodothyronine [58, 59]. While it is assumed that SIXR has a negative impact on xenograft survival by promoting coagulation activation and adaptive immune response [58], the exact role and mechanisms of SIXR are not fully understood. For orthotopic xHTx specifically, it has been postulated that the exposure to cardiopulmonary bypass has an additional detrimental effect [60]; this hypothesis is not generally accepted [61], however. To attenuate inflammation reactions after orthotopic xHTx,

complement inhibitory drugs (cobra venom factor or C1 esterase inhibitor) and various immunomodulatory drugs (interleukin (IL) 1 and IL6 receptor blockers, tumor necrosis factor (TNF) α inhibitors) have been added to existing immunosuppressive regimens [23, 25–27, 62]. Furthermore, glycocalyx shedding, a surrogate parameter for endothelial dysfunction and inflammation, was only marginal under anti-inflammatory therapy [63]. It remains unclear, however, to what extend each of these anti-inflammatory substances contribute: for instance, IL6 receptor blockers have been shown to bind to baboon but not to pig IL6 receptors. Circulating IL6 - which is increased under treatment with IL6 receptor blockers [64] - could possibly moderate detrimental effects in the graft by activating porcine cells [65].

Another approach to prevent SIXR is the addition of anti-inflammatory transgenes to the porcine donor genome, such as human hemeoxygenase-1 (*hHMOX1*) or human tumor necrosis factor α-induced protein 3 (*hA20*), as was done in some of the recent studies [24, 26]. Further knowledge is however needed to clarify the potential benefit of these modifications.

Interestingly, cold non-ischemic preservation has also immunomodulatory effects [66]: after 8 h of *ex vivo* hypothermic cardioplegic perfusion, myocardial tissue was significantly immunodepleted, whereas the perfusate displayed a pro-inflammatory cytokine/chemokine pattern; the following heterotopic heart allotransplantation showed reduced leucocyte infiltrations in the transplant, and the graft's viability was improved compared to controls. These results demonstrate a potential beneficial effect of cold non-ischemic preservation beyond sole reduction of ischemia/reperfusion, deserving further research in the xenotransplantation setting.

GROWTH INHIBITION

Extensive overgrowth of the donor heart was first described in a series of heterotopic thoracic pig-to-baboon xHTx experiments [43, 67], and subsequently, in orthotopic xHTx [23]. Graft overgrowth had also been observed after kidney transplantation between species of different body and organ sizes [68]. Besides genetic determination [69] (an intrinsic or donor-specific factor), several extrinsic (recipient-specific) factors have been described to influence cardiac growth after xHTX: nutrition [69], levels of growth hormone (GH), insulin-like growth factor 1 (IGF1) [70], hormones (thyroid hormones, vascular endothelial growth factor, insulin, catecholamines, endothelin, angiotensin), and mechanical stress/strain [71]. Interestingly, extensive cardiac overgrowth did not occur in the heterotopic abdominal pig-to-baboon xHTx model despite intrinsic mismatch of growth rate and organ size [11]: this is possibly due to the lack of relevant afterload in this non-working model (reviewed in [72]), leading to myocardial atrophy [73] and thereby counteracting the intrinsic growth of the graft [74]. By contrast, in the orthotopic model, the juvenile pig heart needs to adapt to an unphysiologically elevated afterload in an adult baboon [75]; elevated afterload is known to trigger myocardial hypertrophy [76, 77].

Untreated, this (mal-)adaptive myocardial hypertrophy leads to a phenomenon termed "xenogeneic Hypertrophic Obstructive Cardiomyopathy" (xHOCM) and eventually to graft dysfunction and graft loss [74]. Two different approaches have been described to prevent cardiac overgrowth: administration of growth inhibitory drugs or genetic modification of the donor animals. The Munich group administered a combination of a mTOR inhibitor (Temsirolimus), antihypertensive drugs (beta-blocker and ACE-Inhibitors) and fast steroid tapering to counteract intrinsic growth and attenuate cardiac remodeling [23]. Cleveland et al. used a similar approach by using an immunosuppressive regime based on rapamycin instead of MMF and fast steroid tapering; they also observed postoperative periods of severe hypertension, which were treated with milrinone and esmolol [27]. In these drug regimens, the mTOR inhibitor seems to be the most important substance: it has been shown to reduce and prevent cardiac hypertrophy in pressure overloaded animals [78, 79] as well as after human cardiac allotransplantation [80, 81]. By contrast, Mohiuddin et al. did not use drugs to inhibit graft growth, but 10fold genetically modified pigs, which lacked growth hormone receptors (*GHR-KO*) [26]; pigs with *GHR-KO* have been shown to grow slower and smaller than wild-type German Landrace pigs [82]. It has been proposed that a reduction of local myocardial IGF-1, produced by syngeneic resident macrophages, may play an additional role in inhibiting myocardial hypertrophy after xHTx with *GHR-KO* donor pigs [83, 84]. With growth inhibition, whether by drugs or genetic modifications, survival times of up to 6–9 months were achieved [23, 26, 27], thus emphasizing the tremendous importance of growth control after orthotopic xHTx. Especially for clinical use in adult humans, smaller donor races such as Auckland Island pigs are a future alternative to control growth [85], as undesirable effects associated with growth inhibitory drugs or genetic modifications can be avoided.

It must be noted that also rejection episodes [11, 26], as well as inflammation [74, 86] have been described to cause enlargement of the heart, mediated by myocardial edema, cellular infiltrate and/or hemorrhage. To what extend this contributes to chronic graft overgrowth after orthotopic xHTx is unknown; however, acute myocardial edema due to a rejection episode certainly has the potential to further damage an already hypertrophic xenograft.

AVOIDING PCMV/PCR

First described after pig-to-baboon kidney xenotransplantation [87, 88], infection of porcine donors with porcine cytomegalovirus – in fact a porcine roseolovirus (PCMV/PRV) – has been associated with significantly reduced graft survival after orthotopic pig-to-baboon xHTx [86, 89]. The underlying pathomechanism is still largely unknown, but it is assumed that PCMV/PRV infection causes an increase in levels of IL6, TNFα and tissue plasminogen activator inhibitor (tPA-PAI-1) complexes, suggesting a complete loss of the pro-fibrinolytic properties of endothelial cells, eventually leading to multiorgan

failure of the recipient baboon [86, 90]. It has also been assumed that SIXR could be (at least in part) a reaction to PCMV/PRV [91, 92]. There is no effective antiviral treatment or vaccination, so xHTx of organs from PCMV/PRV infected donors must be strictly avoided (e.g., by motherless rearing [93] and rigorous testing protocols [90, 94]). In a recent retrospective analysis, the donors' PCMV/PCR status did not significantly affect the outcome after orthotopic pig-to-baboon xHTx, with maximum recipient survival after transplantation of PCMV/PCR positive hearts of 225 days [95]. More data is needed, however, to confirm these preliminary data before the policy towards PCMV/PCR should be revisited.

CONCLUSION

In the last 30 years, there has been significant progress in orthotopic pig-to-NHP xHTx, with recipient survival increasing from a few hours in 1994 to several months in 2024. Recent improvements in donor genetics, organ preservation, immunosuppressive and immunomodulatory treatments, donor organ growth inhibition and prevention of PCMV infection have led to consistent graft survival, thereby fulfilling the recommendations of the ISHLT as a prerequisite for a pivotal clinical trial. Some questions still remain, but a clinical application has never been closer than today.

AUTHOR CONTRIBUTIONS

ML designed the paper and wrote most parts of it. MB, MS, and BR contributed written paragraphs and revised the manuscript. All authors approved the final manuscript for publication.

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CONFLICT OF INTEREST

ML and BR are founding members of XTransplant GmbH.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Progress in Porcine Kidney Transplantation to Non-Human Primates

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Renal xenotransplantation has recently made considerable progress in overcoming the barrier to its use in humans. This progress has been made possible owing to the use of preclinical pig-to-primate models. Overall, renal xenotransplantation has long been associated with lower survival rates than that of porcine hearts (mainly due to its life-sustaining nature). However, the use of the latest strains of genetically modified porcine donors, combined with progress in the control of the anti-porcine immune response and coagulation, has now enabled survival of up to 2 years. Although the pig-to-primate combination has long been considered a perfect reflection of the human situation, it has several limitations, particularly in terms of different natural anti-porcine antibodies. This fact, in association with survival prolongation, which is considered a prerequisite, has led some pioneering teams to cross the line of human application. However, use in humans will remain anecdotal, and further progress in renal xenotransplantation will be difficult to achieve without the use of non-human primates, which will remain complementary, particularly with regard to major innovations that have never been tested in humans.

Keywords: xenotransplantation, kidney, genetically modified pigs, nonhuman primates, immunosuppression

INTRODUCTION

Significant progress has been made in xenotransplantation (XT) in recent years with the achievement of the first transplantations of genetically modified pig organs into humans.

The evolution of a very long experimental course has involved the use of animal models such as the hamster-to-rat combination, which was very common in the 1990s [1], with the major drawback of being characterized as "concordant" in the absence of preformed antibodies (Abs). Essentially, these earlier models were discordant with pig-to-non-human primate (NHP) (Old World monkey) models, which allow mimicking the pig-to-human situation with the equivalent existence of natural

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Abbreviations: Ab, antibody; AHXR, acute humoral xenograft rejection; AXR, acute xenograft rejection; CMAH, cytidine monophosphate-N-acetylneuraminic acid hydroxylase; CDC, complement-dependent cytotoxicity; CRP, complement regulatory protein; CVF, cobra venom factor; DKO, double knockout; EPCR, endothelial protein C receptor; Gal, galactose-α-1,3-galactose; HAR, hyperacute rejection; mAb, monoclonal antibody; MMF, mycophenolate mofetil; MST, median survival time; mTg, multi-transgenic; Neu5GC, N-glycolylneuraminic acid; NHP, non-human primate; pCMV, porcine cytomegalovirus; PERV, porcine endogenous retrovirus; rATG, recombinant anti-thymoglobulin; rhEpo, recombinant human erythropoietin; SLA, swine leukocyte antigen; TBM, thrombomodulin; Tg, transgene; TKO, triple knockout; TNFR, tumor necrosis factor receptor; XT, xenotransplantation.

Abs against galactose-α-1,3-galactose (Gal) residues in porcine glycoproteins and glycolipids in NHPs [2].

Through the use of pig-to-NHP models, hyperacute and acute rejection phenomena and their mechanisms have been described. Thus, the first genetically modified porcine organs have been tested and have attracted considerable interest [3]. Among those that were tested, the heart and kidney were the most common organs.

For years, the majority of XT investigators [4, 5] have believed that clinical trials of renal xenotransplantation in humans could be considered once prolonged graft survival with good physiological parameters and low infection and recipient mortality rates were obtained and reasonable immunosuppression was achieved in NHPs.

In this article, we summarize the characteristics of renal XT, focusing not only on the most recent progress achieved in pig-to-NHP models but also on their limitations, with the goal of considering human application to address the issue of organ shortage.

Latest Developments

Survival

For a long time, renal XT researchers have struggled to prolong graft survival, whereas heart survival has progressed from a few months in 2000 [6] to more than 3 years in 2016 [7].

Several characteristics may explain this peculiarity, such as the difference in tissue histology and, most importantly, the sustained nature of the model, which is much more likely to affect the vital prognosis of the recipient.

The use of the first Gal knockout (Gal-KO) pigs as donors helped to prolong survival to almost 3 months in 2 NHP recipients under a specific protocol of tolerance induction with the use of a thymokidney [8]. Finally, Chen et al. reported that this novel generation of genetically modified organs remained subject to Ab-mediated rejection [9], confirming that other xenoantigens (xenoAgs) exist that need to be identified and invalidated.

However, since 2015, considerable progress has been made in terms of the survival time, which has reached more than 2 years [10], mainly because of significant improvements in porcine genome editing and the control of the immune response via immunosuppression.

This long-term survival was achieved by progressive success steps.

Iwase et al. were the first to break the barrier of 100 days of survival, reaching 136 days of survival by using Gal-KO, hCD46, hCD55, hEPCR, hTBM and hCD39 transgenic (Tg) pig kidneys with induction therapy with ATG, rituximab, and cobra venom factor (CVF) and strong maintenance therapy with anti-CD40 monoclonal antibodies (mAbs), rapamycin, steroids, anti-IL6R and anti-TNFR mAbs antiaggregation and anticoagulation [11].

Ma et al. compared groups of pig donors with triple knockout (TKO) with either low or high complement regulatory protein (CRP) expression and immunosuppression based on either CD40 or CD40L blockade and reported survival of up to 316 days in a group of cynomolgus monkeys with TKO/high CRP and CD40L blockade [12].

Kim et al. reported more than 1 year of survival (>400 days) in macaques, with a selection of recipients with low titers of anti-pig Abs and CD4 T-cell depletion associated with anti-CD154-based maintenance [13].

Adams et al. reported survival of more than 500 days in macaques receiving double knockout (DKO) pig kidneys and immunosuppression via C5 and CD154 blockade [14]. Interestingly, in this study, graft survival was inferior with the use of TKO, and the deletion of swine leukocyte antigen (SLA) Class I did not confer any survival benefit.

Recently, while describing the first human decedent models, Anand et al. reported the longest graft survival ever obtained in cynomolgus monkeys, which was more than 2 years (758 days), with the use of TKO, hCD46, hCD55, hTBM, hEPCR, hCD47, hA20 and hHO-1 (TKO-7TG), with or without inactivated porcine endogenous retroviruses (PERVs) [10]. Treatment consisted of T- and B-cell depletion and maintenance therapy with an anti-CD154 mAb, mycophenolate mofetil (MMF), transient tacrolimus and steroids. Interestingly, the authors were able to show a clear benefit of the additional transgenes, as compared with the controls, which had limited survival.

Importantly, these results were obtained mainly in macaques and not in baboons, for which the best XT survival remains more modest, up to approximately 11 months (337 days) [15].

Despite this remarkable progress, it is important to note that this improvement in kidney XT survival may not fully reflect the expected human situation. Indeed, a meta-analysis by Firl et al. comparing the outcomes of 1051 life-sustaining NHP renal allo- and xenotransplants revealed an unexpected minor difference in overall survival between the two situations, with a strict comparison of rhesus monkeys receiving at least 6 months of immunosuppression and Gal-KO organs for XT [16]. Moreover, NHP allotransplant survival appeared to be significantly inferior to that of clinical allotransplants at 6 months, suggesting that clinical renal XT would be better than predicted by preclinical data. This spectacular evolution was due to two main improvements, namely, in genome editing and in immunosuppression, which allowed *in vivo* experiments with NHP models.

Impact of the Improvement in Genome Editing: A Proof of Concept in NHPs

Pig-to-NHP XT initially revealed the impact of the first porcine genetic modification via human complement regulatory protein transgenesis to control hyperacute rejection (HAR) [3]. Thereafter, the advantages of invalidating xenoAgs included the ability to control HAR and delay acute humoral xenograft rejection (AHXR) (see articles by *Galli* and by *Wolf* in this Special Issue of *Transplant International*). However, when using this approach, xenoAg expression differed significantly between humans and NHPs, which was a limiting factor for the model (see below).

Impact of the Improvement of Immunosuppression

These longer kidney xenograft survival times were further improved by using various new combined immunosuppressive regimens, including the administration of more or less rATG,

anti-CD4 and/or anti-CD8 mAbs, anti-CD20 mAbs, anti-CD40 and/or anti-CD154 mAbs, anti-C5 mAbs, MMF, rapamycin or tacrolimus to non-human primates (see article by *Buhler* in this Special Issue of *Transplant International*).

Lessons From the NHP Model

Recipient Selection Based on Low Preformed Anti-Pig IgG Titers

Although the above methods have improved xenograft survival, the presence of anti-pig antibodies in recipients remains an important barrier leading to AHXR.

Similar to humans, NHPs have been shown to have variable levels of anti-pig antibodies that correlate with the occurrence of early AHXR [13, 17, 18].

Xenoantigens and Transgene Expression

The generation of KO animals revealed an unexpected appearance of neoantigens that were reactive to natural Abs in NHPs. To assess whether neoantigens associated with multi-transgenic (mTg) TKO cause rejection in the vascularized thymus and kidney xenotransplantation (VT + K XTx) model, Yamada's group compared in baboons the ability of preformed anti-pig nonGal Abs to bind to Gal-KO PBMCs and TKO and mTg for hCD47, hTBM, hEPCR, HO-1 +/− hCD46 and hCD55 PBMCs. The results revealed that baboons with higher binding titers of preformed IgG to TKO-mTg PBMCs than to Gal-KO PBMCs lost their xenografts due to AHXR at 20 days post-operative days, whereas recipients presenting equivalent binding of preformed Abs to both types of cells maintained their renal function longer without rejection but died of complications [19]. These results suggest that neo-xenoAgs from TKO organs also promote acute xenograft rejection (AXR) [19, 20].

Experiments in NHPs have also revealed that human transgene expression levels may have an impact on graft survival; although donor pigs are clones, they do express variable levels of transgenes with variable phenotypes [21, 22].

Physiology and Metabolism of Life-Supporting Pig Kidney Xenotransplants in NHPs

As previously shown by others [23, 24], Anand et al. confirmed sufficient filtration of metabolites by a single transplanted pig kidney in macaques to compensate for the lack of two native kidneys, similar to clinical renal allotransplantation in humans. Furthermore, renal grafts performed with TKO-7TG+/-RI porcine donors supported long-term survival of up to 758 days, in macaques, with parameters such as serum albumin, serum potassium and blood platelet counts remaining within normal ranges in the majority of cases [10].

Proteinuria is known to occur in pigs but is not pathologically significant, unlike in humans and NHPs.

Recent data from pig-to-NHP kidney xenotransplantation revealed moderate proteinuria, probably due to a controlled recipient immune response that reduced physical stress [23]. Kidney graft function was analyzed in baboons cotransplanted with Gal-KO/hCD47 Tg pig kidneys and a vascularized thymus (VT). The results demonstrated that high expression of hCD47 on graft glomerular cells prevented the development of proteinuria. In parallel, the expression of SIRPα (a natural ligand of CD47) was observed in podocytes from porcine naive kidneys and long-term accepted grafts, whereas it was not detected in podocytes from kidney grafts that developed proteinuria [25].

Long-term survivors with functioning grafts had creatinine and potassium levels within normal ranges; however, the phosphorus levels decreased slowly, and serum calcium levels increased [18, 24], confirming data from previous reports [22, 23]. Anemia was observed in recipients of pig-to-NHP kidney xenotransplants and was probably due to the molecular incompatibility between pig erythropoietin (Epo) and the primate Epo receptor [23]. To compensate for the anemia, recipients were treated with recombinant human erythropoietin (rhEpo), with various outcomes. It was shown that long-term survivors treated with rhEpo had stable hematological values, whereas recipients with early rejection developed severe anemia despite the use of rhEpo [18]. However, the occurrence of anemia is still a matter of debate since, in another study, recipients exhibited stable hemoglobin levels without rhEpo treatment [24].

Zoonoses and Viral Transmission

Zoonotic disease transmission is a public health issue.

The risk of PERV transmission to NHPs has been constantly monitored in pig kidney-to-NHP XT experiments, and no *in vivo* transmission has ever been detected (see the article by *Denner* in this Special Issue of *Transplant International*). The issue of porcine cytomegalovirus (pCMV) is different because the latter is not believed to infect NHPs or humans. However, the risk of pCMV replication in porcine tissue from CMV-positive donors under strong immunosuppression in recipients is real, as proven by Higginbotham et al. [18] and ourselves. In our experience, pCMV replication has also been detected in the blood of some recipients transplanted with pCMV-positive kidney xenografts, and the majority of these xenografts exhibited early cell nuclear dystrophia, which may be related to viral replication and may have contributed to XT rejection [26].

NHP Model Limitations

While Tector et al. reported a beneficial effect of cumulative knockout of 3 major xenoAgs (TKO) on human serum reactivity [27], this effect was slightly different in NHPs because, unlike humans, Old World monkeys express N-glycolylneuraminic acid (Neu5Ac) through the persistent expression of the cytidine monophosphate-N-acetylneuraminic acid hydroxylase (*CMAH*) gene, which is responsible for the hydroxylation of Neu5GC [28].

Interestingly, Old World monkeys have increased IgM and IgG binding reactivity to triple-KO pig cells compared with that to double-KO pig cells, which raises the question of the relevance of TKO in NHPs [29].

In the meta-analysis of life-sustaining renal xenotransplantation in NHPs by Firl, the clinical improvement data from wild-type (WT; MST 15 d) to Gal-KO (MST 23 d) and DKO (MST 35 d) support anecdotal reports of decreased survival of TKO (MST 20 d) donor grafts in NHP xenotransplant recipients [16].

However, this feature must be considered with caution as conflicting publications seem to put it into perspective.

Adams reported long-term survival (up to 439 days) in rhesus macaques transplanted with single Gal-KO/CD55 Tg pig kidneys under immunosuppression based on CD40 and complement blockade, which was comparable to the survival time of DKO or TKO pig organs [24]. Finally, the longest survival in primates (>2 years) was obtained with TKO pig kidneys [10]. In the same study, the authors reported that complement-dependent cytotoxicity (CDC) was greater in cynomolgus macaque sera than in human sera [10].

Regulation and Ethics in Animal Experimentation

Many animal rights organizations oppose the use of animals in research, either pigs or NHPs, because of the risk of disturbing the fundamental species equilibrium, especially at this time of transition to clinical xenotransplantation [22].

Moreover, the cost of experiments with NHPs has recently increased considerably. Taken together, these factors explain why access to these models is now restricted to a very limited number of teams.

CONCLUSION

Although pig-to-NHP XT no longer appears to be a perfect research model, it has brought a tremendous amount of knowledge, with impressive prolongation of graft survival with new immunosuppressive regimens, which has allowed recent human clinical application. However, the transfer of XT to humans remains very rare, making statistical analysis based on experimental groups impossible, contrary to NHPs.

Moreover, xenotransplantation experiments in human patients with brain death (a decedent model) as a relevant model [30] have become a matter of debate and cannot be applied easily in Europe.

Pig-to-NHP models of renal xenotransplantation still represent an important experimental opportunity for the study, especially for patients who are not at vital risk, on dialysis or are at a time of desensitization progression.

AUTHOR CONTRIBUTIONS

GB designed the paper. GB and SL-B contributed to the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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REVIEW
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Pig Lung Xenotransplantation: Barriers on the Road to Clinical Translation

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Lungs remain one of the most difficult solid organs for xenotransplantation, owing to its delicate alveolar capillary barrier and intense crosstalk between innate immunity and coagulation system. Multi-gene-engineered donor pig organs combined with co-stimulation pathway blockade based immunosuppressive regimen have extended xenograft survival in preclinical models using non-human primates (NHP) from hours to weeks. Most recently, the first case of lung xenotransplantation into a brain-dead human recipient was reported, confirming technical feasibility without hyperacute rejection while revealing early inflammatory injury and progressive dysfunction. Key barriers include loss of vascular barrier function, dysregulated coagulation and platelet function driven by porcine-human molecular incompatibilities, and antibody-mediated injury. Preclinical data implicate innate immune activation such as natural killer cells and macrophages. Unlike kidney xenotransplantation, which has achieved stable long-term outcomes in NHPs, lungs require attention to immunogenicity against the "fourth antigen" in triple-knockout (TKO) donors that include the positive crossmatch created by the CMAH deletion when TKO organs are tested in NHP. Although consistent multi-month lung xenograft survival has not yet been achieved in preclinical models, the remaining barriers to clinical translation are being defined. This review delineates lung-specific xeno-immune mechanisms and advances aimed at their mitigation, providing insights necessary for future clinical translation.

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Keywords: xenotransplantation, lung, pig-to-baboon xenotransplantation, non-human primate model, decedent model

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INTRODUCTION

Lung transplantation is the gold standard and final therapeutic option for various types of end-stage chronic pulmonary diseases. However, the persistent global shortage of donor organs remains a critical challenge. Despite the utilization of expanded-criteria donors, including donation after circulatory death, and advancements in organ allocation systems, the waitlist mortality remains alarmingly high at over 28 deaths per 100 person-year [1]. Long waiting time and high waitlist mortality highlight the urgent need for alternative solutions. Xenotransplantation—the transplantation of organs from one species to another—has emerged as a promising alternative to address this unmet need. Recent milestones include pig-to-human heart [2] and kidney [3] xenotransplantation under "compassionate use" circumstances. These breakthroughs leveraged extended graft survival and function achieved in non-human primate (NHP) models using

multi-genetically engineered (GE) pigs and advanced immunosuppressive regimens targeting co-stimulation pathway [4–6].

Lung xenotransplantation presents unique and formidable challenges due to the lung's delicate alveolar architecture, large populations of resident immune cells, and inherent susceptibility to inflammation. Xeno lung differs from allo lung by triggering a host of innate immune injury mechanisms that do not normally play any significant role in allo, amplifying the deleterious consequences of inflammation and injury. Lung injury or inflammation typically leads to loss of vascular barrier function, alveolar flooding, and progressive loss of gas transfer functionality. Specifically, the immediate activation of pig lung macrophages after exposure to human blood, rapid accumulation of human neutrophils, and severe platelet sequestration and activation pose hurdles to achieving even short-term lung xenograft survival in NHP model or during *ex vivo* perfusion with human blood [7–10].

Despite multiple breakthroughs that have enabled improved preclinical results and even clinical translation of kidney and heart xenotransplantation, progress in the lung xenotransplantation has remained comparatively limited [11, 12]. Even with similar gene modifications and further intensified immunosuppressive protocols, preclinical NHP models of lung xenotransplantation (including a recent pig lung in a 'decedent' human) reported survival of only a few days to weeks [7, 13].

This review aims to provide a comprehensive overview of the current state of xeno-lung transplantation, highlighting recent scientific breakthroughs, ongoing challenges, and future directions. By synthesizing findings from preclinical studies and experimental models, this article seeks to inform future research and clinical translation efforts in this rapidly evolving field.

STRUCTURAL VULNERABILITIES AND IMMUNOLOGICAL BARRIERS IN LUNG XENOGRAFTS

The extensive surface area of lung vascular endothelium represents a primary initial target of injury in xenogeneic lung transplantation. This endothelium, intimately associated with the alveolar epithelium via a thin connective tissue layer and basement membrane, is a principal target for preformed anti-pig antibodies, "recipient" innate immune cells such as neutrophils, NK cells, and monocytes, and coagulation pathway component adhesion and activation [11]. The lungs also harbor a specialized immune surveillance network, comprised of tissue-resident macrophages, basophils, eosinophils, and other inflammatory cells that normally participate in lung tissue surveillance. While this system is highly effective in detecting and responding to pathogens, it renders the lungs uniquely susceptible to local inflammatory reactions compared with other transplanted organs. Endothelial activation and injury results in loss of vascular barrier function with alveolar flooding which prevents gas exchange in that lung region, exacerbating graft failure [14, 15]. Our prior research consistently demonstrates that inflammatory responses in both the xenograft and the recipient are significantly more pronounced for lung xenografts compared to other pig organs [5, 11, 16–27].

The intrinsic vulnerability of the lungs to xenogeneic injury arises from the interplay of innate immune pathways and interspecies physiological mismatches. Preformed antibodies, particularly those directed against the α-1,3-galactose (α-Gal) epitope, rapidly bind to donor endothelial cells, triggering complement activation and hyperacute rejection within minutes [28, 29]. This cascade leads to endothelial damage, microvascular thrombosis, and pulmonary edema, culminating in graft failure. While the genetic knockout of the galactosyltransferase enzyme (GalTKO) has effectively eliminated α-Gal-mediated hyperacute rejection in other organs [30, 31], subsequent studies revealed that innate and adaptive immune responses persist [18, 32–35]. These responses target other porcine antigens, including those derived from the N-Glycolylneuraminic acid (Neu5Gc/CMAH) and β-1,4-N-acetyl-galactosaminyltransferase 2 (β4Gal) genes [36]. Lungs from GalTKO.CMAHKO.human CD46 (hCD46) pigs, when perfused *ex vivo* with human blood, showed significant reductions in thrombin generation, thromboxane and histamine release, and pulmonary vascular resistance compared to controls without CMAHKO [37]. This genetic combination delays the onset of pulmonary vascular injury and preserves graft function [37].

Building on these findings, triple-knockout (TKO) pigs (GalTKO.CMAHKO.β4GalKO) have shown further improvements. In a notable *in vivo* experiment, lungs from these genetically engineered pigs supported baboon recipients for 5 days without the need for additional human protective transgenes [7]. In comparison, GalTKO lungs without CMAHKO or β4GalKO typically fail within 24 h and provide only marginal function [7]. While the TKO approach initially appeared to be the optimal and foundational genetic modification for xenotransplantation, subsequent research has revealed a new challenge.

Baboons and other Old World NHP possess antibodies against TKO pig cells, apparently targeting a "fourth xenoantigen" that becomes exposed following CMAHKO [38–40]. Our pilot data also support this finding: *in vivo* experiments using genetically engineered pig with 10 genetic modifications (10GE) (TKO. Growth hormone receptor knockout [GHKO].hCD46.hCD55.human endothelial protein C receptor [hEPCR].human thrombomodulin [hTBM].human heme oxygenase-1 [hHO-1].hCD47) pig lungs elicited high levels of innate immune system activation and systemic inflammation in baboon recipients (unpublished). In contrast, the longest survival observed in our *in vivo* studies (31 days) was achieved using pig lungs from donors with "double knockout" (DKO) (GalTKO.β4GalKO) [7]. These findings suggest that TKO organs—even in the widely used 10GE construct or in pigs with fewer genetic modifications—are insufficiently protected against immune-mediated injury in NHP models.

However, the positive cross-matches against GalTKO + CMAHKO or TKO cells were not observed in human [39, 41].

We believe this phenomenon undermines the predictive accuracy of NHP-based transplant results for clinical outcomes, as these immune challenges are unlikely to occur in human recipients. Consequently, while NHP studies provide valuable insights, their results may underperform in reflecting the true potential of these genetically modified organs in clinical settings, emphasizing the importance of refining genetic constructs and preclinical models including work in human 'decedents'. In August 2025, a Chinese group reported the first experimental single left lung xenotransplantation into a brain-dead 'decedent' human patient, observing the organ's histologic appearance for a 9-day period. By day 1 the posterior >80% of the lung xenograft appeared to be filled with fluid, suggesting rapid loss of barrier function, similar to pig-to-baboon lungs when well-described lung xeno rejection mechanisms are not inhibited. This model, if additionally used to assess life-supporting lung function, offers the potential to yield valuable insights into the current viability of and remaining challenges for clinical lung xeno application [13].

INFLAMMATORY AND THROMBOTIC PATHWAYS IN LUNG XENOTRANSPLANTATION

The sequestration and activation of circulating leukocytes and platelets are hallmark features of lung xenograft injury, uniquely severe compared to other xenografted organs. These processes persist even when antibody binding and complement activation are minimized, indicating that additional adhesive and activation mechanisms play significant roles in the pathogenesis [11]. Porcine endothelial cells are potent activators of human leukocytes, primarily through cytokine elaboration and species-specific incompatibilities in cellular pathways.

Interleukin-8 (IL-8), a key chemoattractant produced by porcine endothelial cells, significantly promotes neutrophil adhesion and rolling on the endothelium [42]. Elevated IL-8 levels observed in *ex vivo* pig lung perfusion models stimulate human neutrophil activation and adhesion, exacerbating pulmonary vascular resistance (PVR) and vascular barrier dysfunction [42]. Additionally, porcine endothelial selectins, such as P- and E-selectin, enhance neutrophil tethering and rolling, further amplifying leukocyte infiltration [43]. Blocking these selectin-mediated interactions with inhibitors like GM1271 (E-selectin) and rPSGL-1 (P-selectin) has shown efficacy in mitigating neutrophil-mediated damage [43].

Platelet activation plays a similarly critical role in lung xenograft injury. Porcine von Willebrand factor (pvWF), expressed and released by porcine endothelial cells, demonstrates abnormal interactions with human glycoprotein Ib (GPIb). Unlike human von Willebrand factor (hvWF), which binds weakly to GPIb under normal conditions and requires high shear stress for activation, pvWF activates quiescent human platelets even under low shear stress. This aberrant interaction leads to pathological platelet aggregation and microvascular thrombosis, significantly contributing to the prothrombotic environment within the xenograft. Such conditions exacerbate vascular occlusion and graft injury, creating a formidable barrier to long-term lung xenograft survival [44].

Donor lung pretreatment with desmopressin (DDAVP), as reported by a Korean group, reduces platelet activation by depleting pvWF from endothelial cells and minimizing its interaction with GPIb [45]. Our *ex vivo* lung perfusion studies further support this approach, demonstrating that pre-depletion of pvWF using DDAVP, combined with the administration of GPIb antagonists, effectively attenuates platelet activation [20]. Moreover, humanizing pvWF by replacing a portion of the gene region encoding the GPIb-binding site with its human analogue in GalTKO.hCD46 pig lungs has been shown to suppress non-physiological human platelet aggregation and sequestration within the pig lung and liver [46]. This genetic modification represents a pivotal advancement, providing a safer and more effective approach to lung xenotransplantation. Importantly, it may also facilitate xenotransplantation applications for other organs and cells, broadening its potential clinical impact.

COAGULATION CASCADE ACTIVATION AND DYSFUNCTIONAL THROMBOREGULATORY MECHANISMS

Coagulation abnormalities are a major contributor to lung xenograft injury, driven by endothelial cell activation or damage, which triggers the coagulation cascade and leads to rapid thrombus formation. Key factors implicated in this process include TBM, EPCR, and TFPI. Although porcine TBM can bind human thrombin to form a thrombomodulin-thrombin complex, its protein C activation efficiency is only 1%–10% that of hTBM [47, 48]. Similarly, porcine EPCR and TFPI, which inhibit extrinsic coagulation pathway factors, exhibit significantly reduced activity compared to their human counterparts [47, 49–52].

Our *ex vivo* perfusion model using human blood demonstrated that expressing hTFPI in GalTKO pig lungs effectively suppressed neutrophil activation and provided protective effects, supporting the hypothesis that human-derived coagulation regulatory factors are critical for mitigating graft injury [10]. Baboon *in vivo* models further revealed that co-expression of hEPCR and hTBM was associated with reduced β-thromboglobulin (βTG) levels, consistent lung survival beyond 12 h, and a higher rate of achieving initial life-supporting xenograft function [7]. However, these advances can only delay the onset of barrier dysfunction and fail to completely prevent it.

Early production of thromboxane and histamine also contributes to loss of barrier function [19]. Treatment with the selective thromboxane inhibitor 1-benzylimidazole (1-BIA) combined with H-2 or non-selective histamine receptor antagonists significantly suppressed PVR elevation and delayed vascular barrier dysfunction [21]. However, the combination of drug regimens targeting inflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-8, and IL-6 have not fully

eliminated these mediators, and barrier dysfunction persists. These findings highlight the need for further investigation into the mechanisms driving coagulation abnormalities and inflammation to develop more effective therapeutic strategies.

COMPLEMENT-MEDIATED LUNG XENOGRAFT INJURY

Complement activation is particularly pronounced in lung xenotransplantation due to the organ's high vascular density and unique susceptibility to immune-mediated injury. To prevent some protection against activation of the complement pathway, human complement regulatory proteins (CPRPs)—including decay accelerating factor (DAF_CD55), membrane cofactor protein (MCP_CD46), and membrane-attack-complex-inhibitory protein (MAC-IP_CD59)—have been introduced into the organ source pigs [53]. (Porcine CPRPs are not very effective at controlling human complement activation due to interspecies molecular incompatibilities and differences in their expression levels on vascular endothelium [54, 55]).

In addition, several pharmacological approaches have been explored to mitigate complement activation in lung xenografts, including the use of C1-esterase inhibitor, soluble complement receptor 1 (sCR1), FUT-175, and depleting agents such as cobra venom factor (CoVF). While these strategies have demonstrated partial success, none has provided a definitive solution [56–58]. To address this challenge, genetic introduction of human complement regulatory proteins (hCPRPs), such as CD46, CD55, and CD59, has been investigated. When combined with DKO or TKO backgrounds, these genetic modifications have shown promising results in preclinical *in vivo* models. Specifically, hCPRPs expression in xenografts has been associated with reduced complement deposition, decreased platelet activation, and delayed graft injury in lungs, as well as in other organs such as the heart and kidneys [19, 59, 60].

SELF-RECOGNITION AND XENOGRAFT INJURY BY MACROPHAGE AND NATURAL KILLER CELL

Cellular immune mechanisms are central to lung xenograft injury, involving macrophages and natural killer (NK) cells. Signal regulatory protein alpha (SIRPα), a key inhibitory receptor expressed on macrophages, plays a crucial role in distinguishing self from non-self. Interaction between SIRPα and its ligand, CD47 prevents autologous phagocytosis [61, 62]. However, in the absence of this interaction, porcine cells become highly susceptible to phagocytosis by human macrophages. Introducing human CD47 into porcine cells significantly reduces this susceptibility and effectively inhibits macrophage-mediated phagocytosis [63, 64]. Yamada et al. further reported that expressing CD47 in porcine lungs extended chimerism after bone marrow transplant and improved xeno-lung recipient survival in baboons [65]. While Watanabe et al. reported up to 10-day survival in baboon recipients using a GalTKO base with hCD47 expression alone, our series using GalTKO.hCD46 backgrounds with hCD47 alone or in combination with other humanized proteins (e.g., hEPCR, hTBM, hCD55, human tissue factor pathway inhibitor [hTFPI], and HO-1) failed to achieve consistent survival beyond 2 days [7]. Anatomical localization of hCD47 within the graft may influence its efficacy, warranting further investigation [66].

Pre-harvest donor macrophage depletion using liposomal clodronate has shown attenuation of acute ischemia reperfusion injury in a mouse lung allotransplant model and prevented endotoxin-induced acute lung injury in pigs showing significantly lower levels of TNF-α, IL-6, and thrombin [67, 68]. When it is applied to xenotransplantation, macrophage depletion significantly attenuates hyperacute rejection in wild-type pigs [22, 69]. In our *in vivo* baboon model, not only the use of liposomal clodronate but also anti-pig antibody absorption and cytokine inhibition were associated with longer xeno-lung recipient survival [7].

In addition to CD47, human CD39 and CD73 have emerged as promising anti-inflammatory mediators. These molecules convert extracellular pro-inflammatory ATP into AMP, reducing inflammation and vascular constriction [70, 71]. Genetically engineering porcine lungs to express human CD39 and CD73 could further suppress inflammation and enhance graft survival by modulating the graft's immune microenvironment.

Natural killer (NK) cells play a dual role in xenograft injury by identifying and lysing non-self cells through both antibody-dependent and independent mechanisms. A critical factor driving NK cell activation in xenografts is the incompatibility between human inhibitory receptors and the porcine major histocompatibility complex (MHC), also known as swine leukocyte antigens (SLA). This weak interaction fails to deliver the necessary inhibitory signals, leaving porcine cells vulnerable to NK cell-mediated destruction [72]. Moreover, the absence of human leukocyte antigen E (HLA-E) on porcine endothelial cells exacerbates NK cell activation by preventing the recognition of negative regulatory signals [73, 74]. In contrast, lungs from GalTKO.hCD46 pigs expressing HLA-E demonstrated substantial protection against NK cell attacks both *in vitro* and in an *in vivo* model, leading to reduced early graft injury and prolonged survival in preclinical models [74–76].

IMMUNOSUPPRESSIVE REGIMEN AND TARGETED DRUG THERAPIES

Optimal immunosuppressive regimens for lung xenotransplantation remain undefined. Building on the promising outcomes of co-stimulation pathway blockade in cardiac and renal allo- and xenotransplantation [2, 5, 77–81], strategies targeting CD154/CD40 and CD28/B7 pathways have been investigated *in vivo* lung xenograft models [7]. While these approaches have shown potential in modulating adaptive anti-xeno immunity, they are insufficient as standalone therapies. Consequently, co-stimulation blockade has been combined with conventional immunosuppressive therapies commonly used in human organ transplantation, including pre-transplant induction

TABLE 1 | Known barriers to durable lung xenograft function, mitigation strategies tested to date, and planned next steps.

Key issue	Key issue	Mitigation strategies deployed to date (genetic/pharmacologic)	Address gaps with next steps
Loss of vascular endothelial barrier function reflects the integrated consequence of these processes	Adaptive immune responses		
	Elicited cellular and humoral immunity against pig antigens	Safe, effective immunosuppressive regimen based on co-stimulation-blockade-based regimen	Verify anti-CD154 levels, test higher doses and additional drug combinations so as to prevent elicited anti-donor antibodies and optimize maintenance immunosuppression
	Innate immune responses		
	Dysregulated coagulation	hTBM/hEPCR/TFPI Thromboxane synthesis inhibitor	Optimize endothelial gene expression levels, evaluate different gene combinations alone and with goal-directed anticoagulation therapy
	Platelet activation and sequestration	Donor vWF depletion with desmopressin Humanized vWF; GPIb blockade	Incorporate humanized vWF into existing multi-GE pig lines Optimize anti-GPIb fab dosing
	Complement activation	hCD46, hCD55, hCD59 and other human transgenes; complement depletion with cobra venom factor transitioning to C1 esterase inhibitor, C3 or C5 inhibitors	Optimize pharmacologic drug dosing algorithms, evaluate C3 and C4 inhibitors Evaluate different combinations of human complement regulatory transgenes at optimized expression levels in donor pig lungs
	Injury mediated by preformed antibody	Carbohydrate xenoantigen deletion: α-Gal/β4GalNT2/CMAH Adsorption of preformed antibody by donor kidney perfusion B-cell depletion with rituximab; splenectomy	Standardize crossmatch gating and non-Gal antibody profiling Evaluate CMAH-intact double-knockout (GalTKO.β4GalKO) lungs with 6 or more human transgenes targeting complement, coagulation, inflammation Plasma cell depletion or inhibition
	Cytokine elaboration	Blockade of IL-6R, IL-8R, TNF-α α1 -proteinase inhibitor	Test IL-1R, IL-33 antagonists as add-on to inhibit cytokine elaboration or block effects Explore cytokine absorption using blood filters
	NK cells	HLA-E transgene to engage human CD94/NKG2A inhibitory signaling	Verify NK functional readouts (degranulation, cytotoxicity) in EVLP/NHP, and molecular evaluation of lung xenografts Evaluate CD38 depletion
	Pulmonary macrophages	hCD47; donor macrophage depletion with liposomal clodronate	Develop strategies to inhibit recipient monocyte/macrophage influx, activation Add human-compatible SIRPα, hCD73 to pig edits
	Neutrophils	Blockade of P/E-selectin/PSGL-1 and Mac-1 (CD11b/CD18)	Validate drug engagement, efficacy to inhibit targeted pathway; develop strategies to inhibit NETs Explore adding hCD200 to pig edits

Abbreviations: β4GalNT2: β-1,4-N-acetyl-galactosaminyltransferase-2; CMAH: cytidine monophosphate-N-acetylneuraminic acid hydroxylase (Neu5Gc); EVLP: ex vivo *lung perfusion; GPIb: glycoprotein Ib; hCD46/hCD47/hCD200: human cluster of differentiation 46, 47, 200; hEPCR: human endothelial protein C receptor; hTBM: human thrombomodulin transgenes; HLA-E: human leukocyte antigen-E; IL: interleukin; KO: knockout; Mac-1: integrin αMβ2; NETs: neutrophil extracellular traps; NK: natural killer; NHP: non-human primate; PSGL-1: P-selectin glycoprotein ligand-1; SIRPα: signal regulatory protein-α; TNF- α: tumor necrosis factor- α; TFPI: tissue factor pathway inhibitor; vWF: von Willebrand factor.*

with antithymocyte globulin (ATG) and anti-CD20 (rituximab), along with maintenance regimens comprising steroids and mycophenolate mofetil (MMF). Additionally, splenectomy is often performed in our *in vivo* models to reduce the reservoir of recipient B-cells available to generate *de novo* anti-pig antibodies as well as spleen-resident plasma cells responsible for elaboration of preformed 'innate' anti-pig antibodies, thus hoping to enhance immunosuppressive regimen efficacy [7].

To address the unique inflammatory mediators that we have observed to be elaborated in association with lung xenografts, various anti-inflammatory agents have been explored. We now consistently include anti-TNF-alpha (etanercept), anti-IL-8 (reparixin), anti-IL-6 receptor (tocilizumab), C1-esterase inhibitors, and alpha1-proteinase inhibitors based on the profile of cytokines we have measured in earlier work. Of note, consistent incorporation of all 4 of these reagents is not by itself sufficient to achieve consistent improvement in xenograft survival or prevention of initial barrier dysfunction [7].

A significant milestone in the field was reported in August 2025, when researchers in China conducted the first lung xenotransplantation in a brain-dead human recipient using a 6GE pig lung (TKO.CD55.CD46.TBM) [13]. The immunosuppressive regimen included induction with ATG and a tacrolimus-based protocol, supplemented with MMF, steroids, anti-IL-2 receptor (basiliximab), anti-C5 (eculizumab), Janus kinase (JAK) inhibitor (tofacitinib), and anti-CD80/86 (belatacept). Within the first 24 h radiologic imaging revealed consolidation of the majority of the lung xenograft. We suspect that resident pig lung macrophage

activation triggered histamine and thromboxane elaboration, contributing to significant edema and alveolar damage. In addition, neutrophils, NK cells, and monocytes likely infiltrated the lungs and caused additional inflammation. While deposition of immunoglobulins suggesting antibody-mediated rejection (AMR) was not clearly observed until day 3 we suspect antibody deposition and complement activation were likely present earlier. The authors suggest that there were signs of improvement in parenchymal damage by day 9, but evidence to support this interpretation we do not find compelling. Although this is a single case report, it highlights two critical priorities for advancing lung xenotransplantation: it is essential to measure lung function in addition to histology in order to accurately predict likely performance of a pig lung xenograft implanted with therapeutic intent; and controlling severe inflammation, including AMR, during the first week post-transplant was not accomplished by the regimen this team tested.

In contrast to lungs, *in vivo* baboon models of cardiac and renal xenotransplantation have demonstrated prolonged graft survival with less intensive immunosuppressive and anti-inflammatory regimens [5, 76–81]. Elucidating the unique vulnerability of lung xenografts to inflammation and rejection by tailoring specific therapies to address them will be pivotal in advancing lung xenotransplantation toward clinical application. In **Table 1** we summarize our view of the remaining major barriers, the strategies we have tested to date, and next steps as we have prioritized them, which we hope will allow us to accomplish consistent long-term lung xenograft survival in our preclinical model and, eventually, in humans.

CONCLUSION

Multi-gene engineering of donor pig and mechanism-based adjuncts have extended experimental lung xenograft survival from hours to days and, in selected NHP models, to weeks of recipient survival. The first pig-to-human lung xenotransplantation in a brain-dead recipient confirmed technical feasibility without hyperacute rejection but underscored persistent lung-specific barriers, notably early vascular-barrier failure, coagulation and platelet dysregulation, and antibody-mediated injury. Evidence from preclinical studies also indicates roles for NK cells and for macrophages in acute injury pathways. Progress toward clinical exploration will need further improvement of outcomes in preclinical models. In addition to evaluation of TKO-based multi-GE pig lungs in decedents, we anticipate that results with DKO (GalTKO.b4GalKO.CMAH-intact) multi-GE pig genotypes in NHP models will better predict clinical performance of lung (and other organ) xenografts. Working in parallel in NHP and decedent models, we hope to facilitate identification of gene and drug combinations that are necessary and sufficient to effectively address and dependably overcome species-specific lung xeno barriers.

AUTHOR CONTRIBUTIONS

LB conceived the topic of the manuscript and ST wrote the first draft. LB and RP revised the manuscript. LB and RP read and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

LB is an employee of Revivicor, a subsidiary of United Therapeutics, a PBC.

The remaining author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GENERATIVE AI STATEMENT

The author(s) declared that generative AI was not used in the creation of this manuscript.

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Xenotransplantation Research -the Nonhuman Primate Model Is Preferable to the Human Decedent Model

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Over the past 40 years, the pig-to-nonhuman primate organ transplantation model has enabled progress in xenotransplantation to be made to the point that we are now carrying out initial US FDA-approved clinical experiments on "compassionate" grounds. More recently, the pig-to-human brain-dead decedent model was introduced with claims that this might replace (or at least augment) the pig-to-NHP model. There are, however, several limitations of the decedent model, most notably the very limited period during which the subject may remain sufficiently metabolically and hemodynamically stable to allow meaningful monitoring of the fate of a pig organ graft. It will be exceedingly difficult to provide the regulatory authorities with data from experiments in which truly prolonged graft function has been monitored, whereas this is already being achieved in the pig-to-NHP model. In view of the complications related to the effects of brain death, the data obtained from xenotransplantation experiments in decedents may provide confusing results. There is a real risk that this may influence the regulatory authorities to become overly cautious in approving formal clinical trials of pig organ xenotransplantation to be initiated. We conclude that experiments in human decedents will be unable to replace studies in pig-to-NHP models.

Keywords: kidney, xenotransplantation, brain death, decedent, nonhuman primate

Point of View

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INTRODUCTION

The pig-to-nonhuman primate (NHP) model has formed the basis for xenotransplantation research for the past 40 years [1]. It is this model that has advanced xenotransplantation to the point today where we have been able to initiate clinical experiments. Without the data accumulated from this model over the past 40 years, little or no progress would have been made. However, there are some researchers today who are disparaging of this model. Furthermore, some of those who are most critical of the model and have introduced the human decedent model have ignored the advances made in the NHP model during the past 20 years. We provide examples below.

When comparing the past and future contributions of the two models, a very important consideration is what information and data the regulatory authorities, e.g., the Food and Drug

Abbreviations: FDA, US Food and Drug Administration; NHP, nonhuman primate; TKO, triple-knockout (pig).

TABLE 1 | Selected studies in the pig-to-nonhuman primate (NHP) and pig-to-human models.

A: Selected *in vitro* studies in the pig-to-human or NHP model
1. Anti-wild-type (WT) pig antibodies develop during the first year of life
2. There is a correlation between IgM level (but not IgG) and complement-dependent cytotoxicity (CDC.).
3. Some adult humans have *no* natural anti-TKO pig antibodies
4. All NHPs have antibodies to TKO pig cells
5. A small percentage of allosensitized humans have antibodies to TKO pig cells
6. Deletion of Gal is associated with a reduced human T cell response
B: Selected *in vivo* studies in the pig-to-NHP model
1. Wild-type (WT) pig organ transplantation results in hyperacute rejection; GTKO/TKO pig organ transplantation does not result in hyperacute rejection
2. Expression of a human complement-regulatory and/or coagulation-regulatory protein extends pig graft survival
3. A systemic inflammatory response has been documented
4. Sensitization to pig antigens is *not* detrimental to subsequent allotransplantation
5. CD40/CD154 co-stimulation pathway blockade is superior to conventional therapy, e.g., tacrolimus-based, in an immunosuppressive regimen
6. After pig kidney transplantation, pig renal function has some differences from native NHP renal function

Administration (FDA) in the USA, require before they approve of formal clinical trials of xenotransplantation. With regard to pig kidney xenotransplantation, to our knowledge the US FDA requires evidence of "consistent" survival of pig kidneys transplanted into primates for 12 months. By "consistent," it is uncertain what will be required, but we anticipate that they mean perhaps 6 of 8 consecutive pig kidney grafts being life-supporting for 12 months. Furthermore, the FDA ideally requires that the kidneys should be of the exact pig genotype and the immunosuppressive therapy administered should be identical to that which will be used in the proposed clinical trial. Given the differences in the immune response between NHPs and humans to certain gene-edited pig kidneys, e.g., those from triple-knockout (TKO) pigs [2, 3], the FDA may allow some differences if there is good evidence justifying these.

In view of the challenges of maintaining immunosuppressed NHPs alive and well for a period of 12 months under laboratory conditions (that are primitive in comparison with those in a modern hospital), this will prove challenging. Although the number of NHPs with a pig kidney that have survived for periods >12 months is steadily increasing [4, 5], Kinoshita K, manuscript in preparation), to obtain 6 of 8 consecutive successes will remain difficult. In the light of the results of the small number of clinical experiments being conducted, the FDA may modify its requirement. However, we suggest it will be even more difficult (and expensive) to achieve this in human decedents. Furthermore, the ethics of maintaining a brain-dead human subject under experimental conditions for months may be questioned.

THE PIG-TO-NHP MODEL

The observations made from pig-to-human *in vitro* studies (**Table 1A**) [6] and from pig-to-NHP studies (**Table 1B**) are numerous, and we suggest that, although studies in the pig-to-human decedent model have confirmed several of the observations made in pig-to-NHP models (**Table 2**), nothing truly new has been "discovered" in the decedent model.

Attention has been drawn to the differences in the immune response to TKO pig grafts between humans and NHPs that are related at least in part to the fact that Old World NHPs, like wild-type (i.e., genetically-unmodified) pigs, express the carbohydrate N-glycolylneuraminic acid (Neu5Gc), whereas humans do not [2, 3]. Deletion of expression of Neu5Gc in the pig, as occurs in TKO pigs, appears to result in the expression of another carbohydrate (hitherto unknown) against which NHPs have natural antibodies and strong complement activity [3].

This difference has been put forward by those advocating the human decedent model as a reason why the pig-to-NHP model may not be ideal and does not faithfully mimic the pig-to-human model. However, this major disadvantage of the NHP model has been largely overcome in NHP recipients by (i) selecting NHPs with low anti-TKO pig antibody levels and (ii) immunosuppressing with a CD40/CD154 co-stimulation blockade-based regimen (see below). The fact that pig kidney and heart transplantation in such "sensitized" NHPs can be successful despite this disadvantage surely strengthens the arguments in favor of this model (because there will be many human potential recipients with low levels of anti-TKO pig antibodies [6, 7]).

THE PIG-TO-HUMAN DECEDENT MODEL

This model is limited by several factors [8], of which three are perhaps most important. (i) The short period of time that it may be possible to monitor a pig graft in a stable decedent. This is because brain-dead subjects can become metabolically and hemodynamically unstable, particularly if there is a prolonged period of time between the onset of brain death and the insertion of a xenograft, that may affect graft function and therefore make it difficult to monitor this function accurately. A truly longitudinal study may prove impossible. (ii) There is evidence of a major inflammatory response to brain death that can exacerbate rejection and be detrimental to the induction of immunological tolerance. (iii) In addition, the severe systemic hypertension and cytokine "storm" that can accompany the onset of brain death can result in structural injury and/or cell infiltration in vital organs. At best, the brain-dead model represents a suboptimal environment to assess the response to a pig xenograft.

This can be exemplified by several of the experiments in decedents that have been reported (**Table 3**). The design of some of these was less than ideal, and this undoubtedly contributed to the confusing results that were obtained, making it difficult to determine whether the result was related to the effects of brain death or to the presence of a pig xenograft [9, 10].

One observation made in one of the human decedent experiments was that the pair of pig kidneys that had been

TABLE 2 | Summary of results of pig organ transplantation into human decedents.

Experiment	Organ	Pig Genetics	Immunosuppression	Outcome	What was learned
NYU Case 1	Kidney	GTKO	Reduced conventional	No hyperacute rejection, good urine output, inconclusive function	Confirmed no hyperacute rejection with GTKO pigs
NYU Case 2	Kidney	GTKO	Reduced conventional	No hyperacute rejection, good urine output, focal C4d staining	Confirmed no hyperacute rejection; possible early rejection signs
UAB Case 1	Kidney	10-gene	Conventional	Poor function, TMA, coagulopathy	Confusing results; unclear if due to brain death or immune response
UAB Case 2	Kidney	10-gene	Conventional	Good function, excessive urine output	Pig kidneys can function but with potential complications
NYU Heart 1	Heart	10-gene	Reduced conventional + eculizumab	Successful function for 66 h	Pig hearts can function short-term in decedents
NYU Heart 2	Heart	10-gene	Reduced conventional + eculizumab	Deteriorating function	Variability in outcomes; possible immune response

TABLE 3 | Selected confusing factors in some of the initial experiments in human decedents.[a].

1. Native kidneys were *not* excised (thus making it difficult to ascertain pig kidney graft function)
2. Native kidneys were *not* studied (as 'controls' that had been subjected to the effects of brain death)
3. Kidneys were from GTKO pigs (rather than from pigs with multiple beneficial gene edits)
4. A conventional immunosuppressive regimen was administered (rather than a proven co-stimulation blockade-based regimen)
5. Autologous pig thymic tissue was placed under the kidney capsule. (Did it influence the outcome in any way?)
6. Consumptive coagulopathy developed, and the experiment was terminated for 'exsanguinating hemorrhage'. (was this a consequence of brain death or the presence of a xenograft?)

[a]*Based on Cooper and Kobayashi (2023) (Ref 14, with permission).*

transplanted into the brain-dead recipient produced 37 L of urine in the first 24 h [11]. As, to our knowledge, such a massive diuresis has not been recorded in the pig-to-NHP model and was not seen after pig kidney transplantation into a living patient carried out at the Massachusetts General Hospital in early 2024 [12], this was almost certainly a consequence of diabetes insipidus related to brain death. However, a similar massive diuresis was recorded when chimpanzee kidneys were transplanted into living human patients some 60 years ago, and one patient actually died because fluid replacement could not keep up with the urine output [13]. This type of experience might confuse the regulatory authorities as to the safety of clinical pig kidney xenotransplantation.

In particular, two decisions made by some of the investigators rendered the results much less valuable than they could have been [14].

One was to transplant an organ from a pig with only a single gene edit (α1,3-galactosyltrabnsferase gene knockout, GTKO), a pig that was state-of-the-art in 2004 (20 years ago) but has subsequently been superseded by numerous pigs with multiple gene-edits that more effectively protect the pig organ from the primate immune response [15] (**Figure 1**). If one is serious in wishing to assess whether xenotransplantation is ready for clinical trials, why transplant an organ from a suboptimal pig?

The second was to administer a conventional immunosuppressive regimen (as administered to patients with an allograft), when all the experimental data indicate that a CD40/CD154 co-stimulation blockade-based regimen is preferable [16–18].The argument that the regulatory authorities, e.g., the US FDA, may not approve CD40/CD154 co-stimulation blockade agents for clinical trials is fallacious because the FDA (i) has already approved some of these agents for treatment of other conditions, (ii) has approved them for formal clinical trials of human kidney allotransplantation, (iii) has allowed their use in a growing number of clinical xenotransplantation experiments on "compassionate" grounds, and (iv) has stated that it is accepted that xenotransplantation is unlikely to be truly successful unless this form of therapy is utilized. It has been argued that the human decedent models enable conventional therapy to be assessed, but a study lasting only a few days (or even a few weeks) is surely inadequate on which to come to a conclusion.

A major concern is that, if the results of studies in human decedent models continue to provide confusing results, this may influence the regulatory authorities to become overly cautious in allowing clinical trials of xenotransplantation to proceed. One final question that remains unanswered is whether the immune system weakens with time after brain death has taken place, as it does in the elderly. Is it as vigorous 12 months after the induction of brain death as it is 12 days after the induction of brain death?

The only positive outcomes from decedent studies to date are (i) they confirmed that if an organ from a GTKO or multiple gene-edited pig is transplanted into a human decedent, then hyperacute rejection does not occur (as predicted by numerous *in vitro* studies dating back to 2005), and (ii) they stimulated public interest in the field of xenotransplantation, and drew further attention to its immense potential and, importantly, how close we are to realizing this potential.

FURTHER EXAMPLES OF THE LIMITATIONS OF THE DECEDENT MODEL

1. To fully assess the success of gene-edited pig islet or islet-kidney xenotransplantation, follow-up will be (i) required for

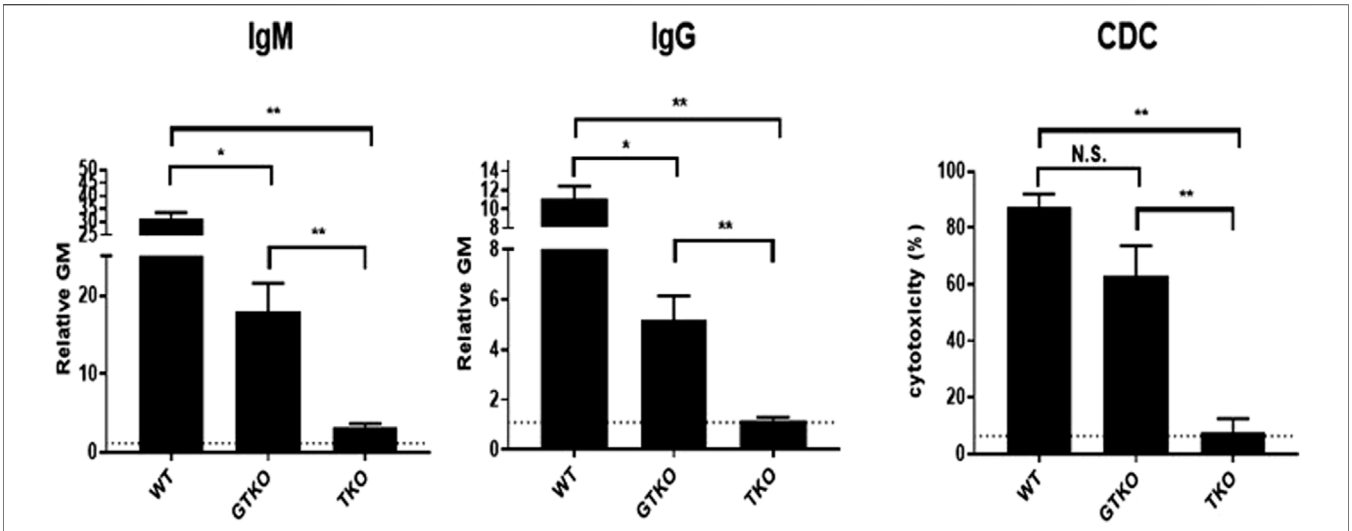


FIGURE 1 | Human serum natural IgM and IgG antibody binding and complement-dependent cytotoxicity (CDC) to wild-type (WT), GTKO, and TKO pig peripheral blood mononuclear cells. The greatly reduced antibody binding and CDC to TKO cells is obvious. (Source: Hara H, et al, 2022, Ref 6, with permission).

several months, (ii) in recipients who are physically active, and (iii) who are taking a normal diet. Is a decedent, who is not moving or eating, a suitable subject for such a study?

2. Orthotopic pig heart transplantation is being explored as a "bridge" to allotransplantation in infants with complex congenital heart disease [19, 20]. Studies in young baboons by our group have demonstrated that (i) a pig heart can sustain a young baboon for >4 months (the median period of time it takes in the USA for a suitable cardiac allograft to become available for an infant) – and, indeed, on occasions for >12 months (Cleveland JD et al, unpublished), and (ii) the replacement of the pig xenograft by an allograft is successful and is unlikely to be associated with immunological problems (Cleveland JD et al. unpublished). Consider how extraordinarily difficult–if not impossible - it would be to carry out this experiment in a human decedent model.
3. Whether the induction of immunological tolerance to a xenograft is to be achieved by hematopoietic cell chimerism or thymic transplantation, it usually requires significant pre-transplant immune modulation, e.g., irradiation or chemotherapy. Can this be tested effectively in decedents when there is little time to prepare the recipient before hemodynamic instability may occur? Furthermore, it may take many weeks or months to develop tolerance, which will require a prolonged period of follow-up. Will this be possible in a decedent?

Many other examples could be given of the difficulties of carrying out these experiments in decedents. In contrast, they are relatively straightforward in the pig-to-NHP model.

There is, however, one possible topic that might usefully be explored in human decedents. The literature suggests that highly allosensitized patients who do not have antibodies that cross-react with TKO pig cell antigens would *not* be at risk of early TKO pig organ graft rejection [21]. The transplantation of a TKO pig kidney into an allosensitized, immunosuppressed human decedent (who did not have cross-reacting antibodies) might prove that this assumption is correct. The result could, of course, be compared with that of a TKO pig kidney grafted into a highly-sensitized decedent who *did* have cross-reacting antibodies.

COMMENT

We suggest that we have enough data from research in NHPs to initiate a meaningful and ethical clinical trial of pig kidney transplantation today [22], and we are close to undertaking a clinical trial of pig cardiac transplantation in infants. What is now required is to carry out an increasing number of pig organ transplants in living human patients, not in human decedents, which we suggest would be a distraction. If there is a need to investigate organ transplantation from (i) pigs with novel gene edits, or (ii) in recipients who will receive an immunosuppressive regimen that includes truly novel agents or a novel tolerance-inducing regimen, this can only be satisfactorily achieved in a pig-to-NHP model (with prolonged follow-up) but *not* in a decedent model.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

DC conceived the topic of the manuscript and wrote the first draft. JC, JS, and DC revised the manuscript. LM reviewed the literature

and compiled **Table 2**. In view of the data presented in this new table, she also revised the manuscript, she read and approved the final version of the manuscript.

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CONFLICT OF INTEREST

DC, JC, and DC are consultants to eGenesis Bio, Cambridge, MA, United States, but the views expressed are those of the authors and not necessarily those of eGenesis.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GENERATIVE AI STATEMENT

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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The Future of Pig Liver Xenotransplantation

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Recent technological advances in genetic engineering have led to a renewed interest in xenotransplantation. Milestones achievements in heart, kidney and more recently liver xenotransplantation raise the hope of an alternative source of organs and pave the way for first in human clinical trials. With these rapid developments, it is important to consider what may be the place of liver xenotransplantation in the treatment armamentarium and what are the challenges to achieve clinical liver xenotransplantation.

Keywords: liver transplant, xenotransplantation, cross circulation, auxiliary liver transplantation, decedent model

INTRODUCTION

Liver transplantation is the curative treatment for end-stage liver failure of many etiologies. The limited availability of human organs suitable for transplantation is, however, a significant limiting factor worldwide. Therefore, heart, kidney and liver xenotransplantation, as an alternative source of organs has been explored with a focus on pigs as the primary species for such transplants due to their anatomical and physiological similarities to humans, and relatively permissive ethical considerations. Recent technological advances in genetic engineering have enabled reliable gene modifications to wild-type pigs to abrogate the known glycan xenoantigens (alpha-Gal, Neu5Gc, and SDa) responsible for preformed antibody mediated hyperacute rejection in humans and other primates [1]. Further introduction of multiple human transgenes has enabled the transplantation of hearts as reported by Griffith et al. into a living human [2]. Subsequently, kidneys and liver xenotransplantation into selected living human patients, under single patient expanded access authorization or compassionate use, have been undertaken and detailed peer-reviewed reports from these recent endeavours are greatly anticipated.

Regardless of the significant advancements in genetic engineering and improved surgical techniques, xenotransplantation of the liver shows inferior outcomes in nonhuman primate studies compared to other organs. Whilst the heart and kidney xenotransplantation survival is measured in years [3, 4], the longest survival of a pig liver-to-baboon is still 29 days as reported in 2017, with a requirement for continuous infusion of human coagulation factors [5]. The liver's complex physiology and the multifaceted challenges of replicating or replacing its functions may have contributed to this discrepancy.

POTENTIAL ROLES FOR LIVER XENOTRANSPLANTATION

With these rapid developments, it is important to consider what may be the place of liver xenotransplantation in the treatment armamentarium and what are the challenges to achieve this. Whilst life-sustaining orthotopic liver xenotransplantation for chronic liver disease indications poses significant challenges that are yet to be overcome, a liver xenograft holds the potential to serve as a bridge to an allograft liver transplant. This could be undertaken either as a full organ transplant

Point of View

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following native hepatectomy for well-defined scenarios or, more feasibly, as an auxiliary support in cases of acute failure of the native liver. In the latter case, auxiliary xenograft implantation of relatively small porcine livers will not interfere with the vascular supply of the native liver, would allow time for the native liver to regenerate adequately and resume its normal functions and would enable a swift subsequent removal of the bridging xenograft. However, the impact of a xenograft on the liver's natural regenerative processes is yet unclear. It is uncertain whether the xenograft would inhibit the regeneration of the patient's native liver or if it would itself experience growth. Indeed, both scenarios present complex challenges that require additional investigation.

The development of *ex vivo* perfusion devices offers a promising solution for the use of xenografts as liver support, removing the need for surgical implantation and acting as an external cross-circulation system that provides critical hepatic metabolic functions and synthesis of vital proteins including coagulation factors during the acute injury phase. This approach is particularly beneficial in cases of acute liver failure, where brief, temporary support is needed. It is yet unclear if the extracorporeal machine perfusion approach may also minimise cellular interaction between the xenograft and patient, thus perhaps reducing the risk of rejection.

CURRENT STATUS

Recently, several milestone achievements in the liver xenotransplantation field were reported. In January 2024, a team from the University of Pennsylvania (UPenn) announced the first use of extracorporeal xenograft liver circulation in a decedent human recipient following brain death. The recipient was connected to a genetically engineered pig liver, developed by eGenesis Inc., which overexpressed seven human transgenes in addition to knock-out of three glycan xenoantigens, and inactivated PERV elements through three rounds of editing and cloning. This was the same genetic background that was recently reported to maintain kidney xenografts for up to 2 years in a nonhuman primate model was used [4]. The OrganOx® machine perfusion device sustained the xenograft, and over a 72-hour period, there was no evidence of rejection, as reported by the investigators to the news outlets [5] but the scientific report and evidence and the final assessment are yet to be published. The trial was electively concluded as planned. It is important to note that the recipient had an intact and functioning liver, which may blur the potential compatibility and function of porcine proteins in humans. Although the preliminary findings are promising and the proof of concept was demonstrated, particularly considering the significant challenges observed in nonhuman primate studies, the short duration of the trial means that further research is necessary to reach definitive conclusions with regards to functional support.

In March 2024, a team from Xijing, China announced the implantation of a genetically modified porcine liver containing six genetic modifications, that deleted three glycan xenoantigens and introduced three genes for human proteins, into a decedent recipient. The liver xenograft was observed for 10 days, and the group report no gross evidence of rejection. Two hours after portal vein reperfusion of the xenograft, bile production was noted. Porcine liver-derived albumin increased after surgery and alanine aminotransferase levels remained in the normal range. Blood flow velocity in the porcine hepatic artery and portal and hepatic veins remained at an acceptable level [6]. Similar to the UPenn report, the recipient liver was not removed, hindering a full evaluation of the pig liver functions. A combined liver-kidney transplant has also been undertaken in a decedent model in China, demonstrating the feasibility of the procedure although details are still awaited.

On 17th of May 2024, a team from the First Affiliated Hospital of Anhui Medical University reported a liver xenotransplantation into a living human patient. The recipient, a 71-year-old man diagnosed with a liver tumour underwent right hemi-hepatectomy followed by an auxiliary liver xenotransplantation due to insufficient size of the remnant left liver. The pig liver had 10 genetic edits (three to delete the glycan xenoantigens and 7 to induce production of human proteins) engineered at the Yunnan Agricultural University in Kunming, China. Early reports of the patient's recovery were favourable and further updates are awaited.

PRESENT CHALLENGES AND PROSPECTS FOR CLINICAL APPLICATION

There remain numerous immunological barriers for all types of pig-to-human xenotransplantation and these are particularly important for liver transplantation. Although immediate hyperacute rejection has diminished with the advance of alpha-Gal knock-out pigs, many other factors contributing to graft failure remain.

The introduction of novel immunosuppressive drugs including anti-CD154 monoclonal antibodies has offered some benefits for the successful outcome of xenotransplantation as evidenced by pre-clinical animal studies [6, 7] and as such the clinical use of an anti-CD154 monoclonal antibody is likely to play a significant role in any future application of xenotransplantation.

Thrombocytopenia occurring without signs of a rejection reaction, indicates alternative mechanisms and further molecular incompatibilities. Compared to human livers, porcine livers have reduced albumin production [8]. Additionally, pig thrombomodulin does not interact correctly with human Protein C receptor. These are among multiple recognized incompatibilities being addressed by genetic engineering and introduction of human transgenes.

Unlike other organs such as the heart and kidneys, the liver has multiple complex functions, both detoxifying harmful compounds and synthesizing a wide range of proteins that play a vital role in both hepatic and systemic processes. While there are parallels between pig and human physiology, considerable differences exist. Conserved functions for detoxifying compounds absorbed via the enteric system may be similar, yet the exact metabolites produced by these processes may vary. Moreover, as seen in human liver pathology, abnormal folding or genetic mutations of proteins may lead to storage diseases and accumulation of faulty proteins causing injury both in the liver

and elsewhere. These potential pathologies need to be considered and monitored, especially since they tend to manifest after several years of accumulation. These long-term effects are not apparent in animal studies and could not be assessed in the groundbreaking work undertaken so far. Thus, patient selection and a clearly defined anticipated benefit for the first in human trials are critical.

The genetic modifications in engineered pig organs are specifically optimized for compatibility with human immune systems, which suggests that data from nonhuman primates may not reflect the full potential anticipated in humans. Furthermore, this implies that the outcomes in humans may surpass the results seen in animal models.

Zoonotic transmission remains a major hurdle in xenotransplantation compounding the infectious complications already inherent to organ transplantation. Porcine cytomegalovirus has emerged as a difficult to test and treat virus in this context. While it may not infect the human recipient, it may injure the xenograft causing potential failure. This mechanism is believed to have contributed to the dysfunction of the first heart xenotransplantation.

Although still debated, xenogeneic organs, when transplanted into recipients, are expected not to provoke the production of alloreactive antibodies. Consequently, they appear to not sensitize the recipient for future allogenic transplantations. This is particularly important in auxiliary xenotransplantation, where liver xenotransplantation serves as a bridge to subsequent allograft transplantation [9, 10].

Xenotransplantation, including liver xenotransplantation represents a promising Frontier in transplantation medicine, offering hope for addressing the dire shortage of human donor organs. The field has witnessed significant progress, with the recent decedent studies and the successful report of a living human recipient marking a pivotal moment in its development.

Clinical trials may well be the next Frontier, but the challenges ahead are significant, including immunological barriers [11], longitudinal effects, regulatory restrictions and a clear definition of the foreseen role in the management of chronic or acute liver failure. To reassure the public and ensure safety, it is paramount that current genetic engineering and breeding are undertaken under DPF (Designated Pathogen-Free) conditions to ensure that donor pigs have a very high safety profile. Moreover, it is essential to maintain a dialogue with the public, ensuring that the societal implications of xenotransplantation are considered alongside the scientific advancements [12]. To achieve this, it is paramount that Europe re-engages in xenotransplantation and some of the clinical experimental work and indeed clinical trials are undertaken here.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Opinions on the Future of Clinical Pig Kidney Xenotransplantation

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Based on promising results obtained in primate models, pioneers in the US have now started to explore the new frontier of genetically-edited pig-to-human transplantation. The recent transition of xenotransplantation into clinical medicine has included transplants in brain-dead subjects and the compassionate use of xenotransplants in living recipients without options for allotransplantation. While the barrier of hyperacute rejection seems to be successfully overcome by gene editing of donor pigs, the occurrence of accelerated rejection could pose significant limitations to the success of the procedure. Ultimately, the establishment of efficient and safe strategies to overcome immunologic barriers will, among other critical factors, such as potential xenozoonotic disease transmission or physiological differences, determine whether and for which indications xenotransplantation will be viable. Considering preliminary outcomes of compassionate use xenotransplantions, which may raise questions about how faithfully data from non-human primate models translate into human outcomes, further research in decedents may be necessary before proceeding with additional clinical transplants. Looking ahead, designing systematic trials in xenotransplantation, including the definition of acceptable eligibility criteria for such high-risk transplants, will be an immense challenge, especially in kidney transplantation, where dialysis provides an effective alternative to transplantation in most cases.

Keywords: clinical transplantation, decedent model, gene-edited pigs, rejection, trial design

Point of View

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While the authors of this article have no experience in xenotransplantation, neither in experimental research nor in clinical pig-to-human transplants, they have a longstanding scientific interest in strategies for the prevention and treatment of antibody-mediated rejection (AMR) in the context of allotransplantation. Consequently, immunologic aspects—highly relevant also in allotransplantation—dominate their considerations. They are aware that even if the immense immunologic xenobarriers can be successfully managed, other significant factors, such as associated infectious risks, differences in physiology, and important ethical considerations, could come to the forefront [1–3]. Nevertheless, despite these profound challenges, there are sufficient arguments to maximize research efforts towards alternatives to allotransplantation, which, given the speed of development, may include pig-to-human xenotransplantation. Organ transplantation faces an immense organ shortage, posing a considerable burden on the disadvantaged patient group with end-stage organ failure. The transplantation of genetically modified pig organs into humans could potentially fill this gap, avoiding unacceptable waiting times and reducing death rates on waiting lists.

Thanks to major advances in genetic engineering and the establishment of efficient immunosuppressive strategies, recent studies have achieved long-term, rejection-free renal

xenograft survival in non-human primates (NHP) [4]. This success was achieved even without the use of CD40/CD154 costimulatory blockade, a strategy shown to reduce immunological risks in xenotransplantation, but not yet approved by the Food and Drug Administration (FDA) for human use [5]. Beneficial long-term results obtained in primate models have finally led pioneers to explore the new frontier of genetically-edited pig-to-human transplantation. We are now witnessing the transition of this concept into clinical medicine, including experimental studies in brain-dead human subjects [6–11], as well as preliminary clinical experiences with pig-to-human xenotransplantation, including heart, kidney, and even liver transplants [12–15].

Actual data on the first human xenotransplants suggest that immunological factors, particularly the occurrence of AMR, are critical in limiting the success of the procedure [16–18]. It could be argued that our ability to establish efficient and safe strategies to overcome immunologic barriers will be decisive in determining whether and for which indications xenotransplantation will be viable. Currently, we do not know how human recipients will respond immunologically to the immense burden of xenoantigen mismatches in the intermediate term, let alone over a long period. Even in short-term experiments, the multifaceted interplay between different components of innate and specific immunity in this context remains poorly defined.

Several centers have now studied porcine xenografts in decedents to explore aspects of immunology, coagulation, infectious disease, and metabolic functions. Using different variants of genetically modified pig kidneys, it has been demonstrated that pig xenotransplants can maintain renal function and provide physiological balance for days up to several weeks [6–11]. A major breakthrough from all these experiments was the effective prevention of hyperacute rejection through genetic modification of donor pigs. Remarkably, while several xenotransplants were performed using pigs with a considerable number of genetic edits, this was achieved also with thymokidneys from pigs with only the alpha-1, 3-galactosyltransferase antigen knocked out [7]. Although the prevention of hyperacute rejection represents a significant step towards successful xenotransplantation, detailed analyses of renal xenotransplants in deceased subjects revealed molecular and morphological features of AMR just a few days after transplantation [17]. In some cases, possibly triggered by a decedent systemic inflammatory process, the occurrence of microvascular injury in the form of thrombotic microangiopathy was observed [6], with recent data supporting utilization of complement inhibition at C5 to control the innate human immune response to porcine kidney xenografts [19].

As part of compassionate use, xenotransplants have been performed in patients with end-stage organ failure. Following two clinical heart xenotransplants in 2022 and 2023, two subsequent clinical kidney xenotransplants were conducted in critically ill patients without alternative options, according to the literature [12, 14]. The first kidney xenotransplant, performed in Boston in March 2024, utilized a gene-edited pig kidney with 69 genomic edits to address immune and coagulation incompatibilities and inactivate porcine endogenous retroviruses. Immunosuppression included costimulatory blockade and complement inhibition. The recipient, however, died in less than 2 months, possibly due to poor underlying health, but data describing the exact circumstances are not yet publicly available [14]. A second kidney xenotransplant at NYU Langone Medical Center in New York City involved a 54-year-old woman with heart and kidney failure. This procedure included a dual transplant of a left ventricular assist device and a gene-edited thymokidney xenograft in a living recipient. The kidney was explanted after several months, but detailed results have not yet been published in a peer-reviewed journal. The latest results from compassionate use clinical transplants—where all recipients (two heart and two kidney) have either died or lost their transplants—raise questions about how accurately data from NHP models translate into human outcomes, where we aim for long-term organ replacement. The authors of this article share the opinion that these results underscore the need for further research before proceeding with additional clinical experiments in living transplant recipients [11].

But how to move forward? After more than 30 years of research with non-human primates, the obvious limitations of this approach are extensively discussed [11]. Likewise, the use of brain-dead decedents raises ethical concerns with a high emotional burden on relatives and staff. But also costs and medical issues due to brain death with all its potential confounding factors restrict the use of experiments involving decedents to short periods. The longest experiment with a decedent was terminated after 2 months and included the reversal of xenograft rejection using multimodal treatment including complement blocking agents [11]. While this case may indicate some incremental achievement, it also demonstrates the difficulties to overcome the strong immunological barriers just for a few weeks. The freedom from AMR months after transplantation in a small cohort of decedents could represent a significant milestone before advancing to living recipients. However, such a requirement will be difficult to achieve in a decedent model due to ethical concerns, costs and long observation time.

Preliminary results indicating a role of early AMR are critically important, particularly given the lack of approved effective treatments to counteract antibody-triggered graft injury even in clinical allotransplantation, where, chronic rejection, often preceded by acute AMR, has emerged as a major cause of accelerated organ loss [20]. We do not know whether new treatments showing promise in allotransplantation will also succeed in xenotransplantation, given the significant genetic disparity between recipient and donor and qualitative differences in rejection processes. However, the xenotransplantation field could benefit from the increased understanding of rejection pathophysiology, particularly the recognized major role of NK cells, which has led to new developments in anti-rejection treatments [21]. For instance, a recent phase 2 trial using a human antibody targeting CD38 showed effective reversal of donor-specific antibody-associated microvascular inflammation, likely achieved through selective depletion of NK cells expressing Fc gamma receptor IIIA

[22]. The next steps will be to evaluate whether such innovative treatments could also counteract xenotransplant rejection. It could be argued that the absence of recipient HLA on pig cells may trigger a robust NK cell "missing self" response, which could potentially be addressed by targeting CD38. The role of NK cells in rejection of allo- or xenotransplants, which may involve antibody-independent effector mechanisms, could be significant. However, despite existing therapeutic concepts, the precise pathways underlying DSA-independent injury have yet to be fully defined. This is especially important in xenotransplantation, where various effectors may contribute to intermediate- and long-term xenograft damage. Given that the two primary challenges in transplantation are access to transplants and chronic AMR—the latter a major limitation to long-term allograft survival [23]— it is crucial that these aspects be integrated into xenotransplant research with both urgency and importance.

A key question will be how to progress xenotransplantation to systematic clinical trials beyond compassionate use in isolated patients, particularly considering that initial transplants have not been as successful as anticipated based on primate studies. In the US, individual transplant cases have been conducted with FDA permission through the Expanded Access pathway, including two cardiac and two kidney xenotransplants [24]. For this pathway, three conditions must be met: the patient has a life-threatening illness; there is no therapeutic alternative; and the benefit-risk ratio is favorable. While it is surprising that these authorizations were issued without a clear understanding of the safety and efficacy, this strategy may be useful to gather more data to support future trials [24]. Detailed information on some xenotransplants are not yet available, but this information is crucial for the continuation of clinical transplants. Therefore, transparency and prompt publication of details on failed cases are indispensable prerequisites for progress. However, successful transplantation in patients who are too sick to be listed on a regular waitlist will be difficult. Given the current results, designing trials in xenotransplantation presents immense challenges and multidisciplinary collaborative efforts are needed to overcome these hurdles and develop an ethical path forward towards first studies [25]. The patients who willingly accept the risks associated with participating in xenotransplant trials, which could potentially include accelerated death, will be true heroes. However, how much risk can we ethically allow participants to bear in a trial, and what should the appropriate eligibility criteria be? For heart transplants, it may be relatively straightforward to define eligible cohorts, such as patients with terminal heart failure who are unsuitable for mechanical devices and have a very limited life expectancy. For such patients without a real alternative, even a few months of additional life expectancy may create a benefit. Additional considerations for patient with terminal heart failure are the option of a xenograft as potential bridge to a suitable human heart.

However, the situation is significantly more complex for kidney transplants, as dialysis serves as an effective replacement therapy in the majority of cases, and we would expect a transplant ideally to function for decades and not just a few months. The balance between transplantation and the life-saving but often suboptimal option of long-term dialysis remains a critical issue. It is well established that—depending on donor characteristics, recipient age and the type and severity of underlying medical conditions—the decision to maintain a patient on dialysis versus proceeding with a transplant has a significant impact on patient survival [26]. Any bridging strategy (e.g., xenograft until availability of a suitable human allograft) has also to consider the risks of heavy immunosuppression. A recent European consensus paper suggested including high-risk hemodialysis patients with poor predicted survival or those experiencing difficulties with dialysis access [27]. However, even for these patients, allografts may be available through high urgency listing with 80% patient and 70% graft survival at 5 years [28]. Another consideration could involve including extremely sensitized patients who have no realistic chance of receiving an allograft. Nevertheless, specific allocation programs, like the acceptable mismatch program, along with new therapeutic options such as imlifidase and targeting CD38, could potentially pave the way for successful allotransplantation in such cases [29, 30]. However, even innovative treatments come with inherent limitations. For example, when discussing imlifidase, it is important to note that despite its high efficiency in transient antibody depletion, a major limitation is the rapid reconstitution of HLA antibodies, which restricts its utility in desensitization and treatment protocols [31, 32]. Additionally, it is challenging to predict whether and to what extent a previous xenotransplantation would increase rejection risks in highly alloimmunized patients. A recent model using xenokidneys expressing seven different human transgenes in highly allosensitized rhesus macaques, combined with anti-CD154 monoclonal antibody-based immunosuppression, suggested that prolonged graft survival might be achievable in this high-risk population without the risk of hyperacute rejection triggered by alloantibodies [33]. In this model, allosensitization via serial skin transplantations only temporarily elevated xenoantibodies, and the authors did not observe an increase in alloantibodies post-xenotransplantation or xenograft rejection [33]. Such data are encouraging. However, careful interpretation is warranted, as we have learned that primate data may not necessarily translate to the unique challenges of pig-to-human transplantation.

The clinical trial design in xenotransplantation may not follow conventional rules, and it may be prudent to significantly limit the sample size, especially in the initial phase. For instance, a strategy of sentinel groups, comprising a few treated patients at the outset of the trial before expanding to more participants, may be appropriate. Initial trials must be informed by comprehensive data from preliminary (pre)clinical experiments. This includes establishing the immunosuppressive regimen, covering both induction therapy and maintenance immunosuppression. Additionally, a clearly defined arsenal of rejection treatments is essential, along with diagnostic procedures to guide treatment. This may involve gene expression analyses and the use of biomarkers, such as donor-derived cell-free DNA. The sample size and trial design will require thorough discussion, potentially necessitating an uncontrolled trial setting, including comparisons with matched control groups on dialysis.

Ultimately, what is the primary endpoint and the goal of future clinical trials? As an intermediate goal, a lifesaving bridging strategy may be a reasonable approach to explore the feasibility of a xenograft for a limited period, with the option of a long-term life-sustaining human allograft. The outcomes of such initial trials will determine whether xenotransplantation holds promise as a bridging therapy and could be gradually extended. Their initial results will also critically impact the acceptance of xenotransplantation by patients and the public. Ideally, collaboration among various groups experienced in experimental xenotransplantation, working closely with transplant clinicians involved in highly sensitized transplant programs, and supported by innovative companies advancing gene editing, will be crucial for conducting systematic trials. Let us hope that such collaborative efforts successfully navigate the challenges ahead!

Medical pioneering often encounters setbacks, as exemplified in the initial phase of clinical allotransplantation, where kidney transplantations performed in the 1950s were mostly discouraging and seriously questioned clinical applicability [34]. Nevertheless, a decade later, owing to technical advances and developments in immunosuppression, allotransplantation became a clinical standard of care. The history of transplant medicine underscores how difficult it is to foresee medical triumphs based on initial cases, especially given that progress in medical research typically does not follow a predictable linear path [35]. Undoubtedly, xenotransplantation has made considerable progress over the last 30 years moving from hyperacute rejection minutes after transplant to potentially treatable xenograft rejection after weeks, but there is still a long way to achieve transplant success for decades. We want to conclude with a remark from Sir Roy Calne who, in 1995, said that xenotransplantation "is just around the corner, but it may be a very long corner" [36].

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Pig Xenotransplantation in Beta Cell Replacement: Addressing Challenges and Harnessing Potential for Type 1 Diabetes Therapy

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This opinion paper evaluates the potential of porcine islets as a promising alternative in beta cell replacement therapy for Type 1 Diabetes (T1D), juxtaposed with the current limitations of human donor islets. It analyzes the compatibility of pig islets with human glucose metabolism, their prospects as a limitless and high-quality source of beta cells, and the unique immunogenic challenges they present in xenotransplantation. Additionally, the paper discusses the regulatory and ethical considerations pertinent to the use of porcine islets. By synthesizing current research and expert perspectives, the paper highlights both the opportunities and significant barriers that need addressing to advance pig islets as a viable therapeutic option. The findings advocate for a balanced and forward-looking approach to the integration of pig islets in T1D treatment, underscoring the need for continued research and dialogue in this evolving field.

Keywords: porcine islets, xenotransplantation, type 1 diabetes, immunogenicity, regulatory framework

INTRODUCTION

The path to curing Type 1 Diabetes (T1D) through beta cell replacement is filled with both promise and complexity [1, 2]. At its core, the strategy is simple and compelling: by restoring the insulin-producing function of pancreatic beta cells, one can tackle T1D at its root [3]. The use of islets from organ donors in these therapies has convincingly shown that such an approach can significantly improve or even temporarily reverse diabetes [4–8]. Yet, this achievement also highlights the critical hurdles that need to be cleared to reach a universal cure. The main challenges in beta cell replacement therapies involve finding a limitless supply of beta cells, reducing or avoiding the need for immunosuppressive drugs, and ensuring the transplanted cells survive and function over the long term [9]. The reliance on donor islets is severely limited by donor scarcity, variable islet quality, and the complexities tied to working with primary culture cells, which together pose a significant challenge to the broad applicability of beta cell replacement methods. The pursuit of alternative sources of cells holds potential; however, these alternatives should deliver distinct advantages over current options in terms of availability, quality consistency, and ease of handling to truly transform the landscape of beta cell replacement therapies. Given these foundational challenges, it becomes crucial to consider if pig islets could potentially offer advantages over traditional organ donor sources or alternative strategies, such as those involving the differentiation of pluripotent stem cells [10]. Addressing this issue effectively requires answering some key questions.

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TABLE 1 | Comparative analysis of porcine and human islet function and potential in xenotransplantation.

Aspect	Details	Consequences
Insulin Response [12–16]	Porcine islets secrete 3–6 times less insulin than human islets in response to glucose	May require transplantation of more islets or genetic modification to meet human insulin requirements
Metabolic Control [17, 18]	Pigs have more glucose tolerance and lower basal insulin levels compared to humans	Adjustments in insulin therapy might be necessary post-transplantation to ensure metabolic control
Glucagon Response [19]	Porcine islets show a strong glucagon response to hypoglycemia, beneficial for clinical applications	Could enhance safety by preventing hypoglycemia in recipients
Diabetes Resistance [20–23]	Pigs are resistant to amyloidosis, unlike humans, possibly due to differences in IAPP sequence	Might reduce risk of islet dysfunction and extend longevity of the xenotransplant
Genetic Engineering [18, 24–26]	Human transgenes in pigs do not adversely affect glucose metabolism	Genetic modifications can make xenotransplantation a viable solution without disrupting glucose levels

HOW COMPATIBLE IS THE GLUCOSE METABOLISM REGULATION OF PIG ISLETS WITH HUMAN PHYSIOLOGY FOR TRANSPLANTATION PURPOSES?

The critical prerequisite for evaluating pig islets for transplantation is that the regulation of glucose metabolism by the pig islet must be compatible with human physiology [11]. While pig and human insulin are remarkably similar, differing only in the 30th amino acid of the β-chain, and despite the historical use of porcine insulin in treating human diabetes, there are differences in how porcine islets respond to glucose and other stimuli compared to human islets (summarize in **Table 1**) [12–22, 24–26]. Although the precise long-term consequences of these differences in pig islet function remain uncertain, they seem to align with the requirements for a short to medium-term approach.

IS THE SUPPLY OF INSULIN-PRODUCING CELLS FROM PIG ISLETS A LIMITLESS AND CONSISTENTLY HIGH-QUALITY SOURCE?

Bypassing the obstacles related to genetic adjustments and the circumvention of immune system rejection, the notion of pig islets serving as an endless and consistently superior source of insulin-producing cells encounters multiple critical areas in need of continuous improvement [27]. It is imperative to ensure the uniform quality and longevity of islet cells across various preparations, which mandates the formulation of uniform protocols for the extraction, refinement, and preservation of pig islets to maintain their operational effectiveness and durability post-transplant [28–30]. Additionally, the ability to upscale the production of pig islets poses an essential challenge [31, 32]. This encompasses the initiation of responsible and ethical practices in pig rearing, the advancement of proficient methods for islet extraction, and the creation of effective logistical solutions for their distribution [33]. These factors need to be refined to address the worldwide demand for insulin-producing cells while safeguarding animal wellbeing and ecological integrity. The ongoing debate (summarize in **Table 2**), indicating that after extensive research there still has not been a consensus on the most suitable pig age and strain for providing sufficiently viable isolated islet cells for clinical xenotransplantation [47–50], underscores the current limitations in achieving a limitless and consistent supply [51].

DO PIG ISLET CELLS POSSESS INHERENT ADVANTAGES IN TERMS OF IMMUNOGENICITY FOR XENOTRANSPLANTATION PURPOSES?

In principle, xenotransplantation of pig islets could present a less specific target for the recipient's autoimmune response compared to human islets, due to the differences in cellular antigens between species. Thus, while the pig islet cells could still be recognized as foreign by the recipient's immune system, they might not be specifically targeted by the autoreactive T cells that are involved in the autoimmune attack on native pancreatic beta cells. However, this potential advantage is complicated by the broad immune response against xenogeneic tissue, which includes not only adaptive immune responses [52–56] but also innate responses [57] and issues like the instant blood-mediated inflammatory reaction (IBMIR) [58–63], hyperacute rejection, and acute cellular rejection. These xenogeneic reactions can be strong and present significant barriers to the long-term survival and function of the transplanted islets. Furthermore, cross-reactivity between swine leukocyte antigen (SLA) and human anti-HLA-specific antibodies is another factor that complicates xenotransplantation [64]. It is well-known that human anti-HLA antibodies, especially in sensitized individuals, can sometimes bind to SLA molecules due to structural similarities [65, 66]. This cross-reactivity can lead to both innate and adaptive immune responses, potentially causing early rejection of the pig islets. While the basic concept of cross-reactivity is established and supported by various experimental studies [67–70], the extent to which cross-reactivity impacts clinical outcomes is still debated [71, 72]. The predictability of which specific human antibodies will cross-react with SLA, and how this cross-reactivity might vary among different individuals or pig donors, remains an area of ongoing research [73, 74]. There is also debate about the best

TABLE 2 | Advantages and disadvantages of fetal, neonatal and adult pig islets for clinical xenotransplantation.

Aspect	Fetal pig islet-like cell clusters (ICCs)	Neonatal pig islets (NPIs)	Adult pig islets (APIs)
Source Age	Fetal	<14 days old	>12 weeks old
Isolation and Preparation Challenges [32, 34–37]	Straightforward; includes enzymatic digestion and culture	Comparable to ICCs, with effective recovery thanks to damage resistance	Echoes human islet isolation, accounting for donor pancreas condition, blood removal, and warm ischemia. Density gradient centrifugation with Ficoll and Iodixanol enhances yield and viability
Islet yield/pancreas (Beta cells %) [32, 34, 38]	7,000–10,000 (<10%)	25,000–50,000 (25%)	200,000–500,000 (>70%)
Maturation for *In Vivo* Functionality [39–45]	2–3 months maturity required for *in vivo* functionality	More responsive to glucose, leading to restoration of normoglycemia due to β cell expansion and differentiation	Immediate functionality post-transplantation
Safety	*In vivo* proliferation, minimal tumorigenic risk, low pathogen transmission risk	*In vivo* proliferation, minimal tumorigenic risk, low pathogen transmission risk	No *in vivo* proliferation or tumorigenic risk, low pathogen transmission risk
Cost and Practicality Considerations [31]	Not directly stated	Low cost of maintenance pre-pancreatectomy, simpler isolation, lower costs than APIs, but requires more donors for sufficient islets	High maintenance cost, isolation difficulty and cost, but greater yield of high-quality islets from retired breeders
Pig islet donor for a primate recipient	Not currently being considered for xenotransplantation (because of limited β-cell yield, poor insulin response to glucose and high destruction rate post-transplantation)	Requires a minimum of four neonatal donors per diabetic primate	A single adult donor may be sufficient
Pig islet donor for a human recipient		14–28 donors needed	1–2 donors sufficient
Clinical Application Potential [39, 46]		Limited by the need for multiple donors and additional maturation steps	Significant, with the potential for single-donor clinical transplantation

strategies to mitigate these risks, including whether genetic modifications to reduce SLA expression or to incorporate human-like antigens in pigs are sufficient to prevent cross-reactivity [75]. Thus, while pig islet cells might have certain inherent advantages in terms of reduced targeting by autoreactive T cells, the risk of cross-reactivity with pre-existing anti-HLA antibodies adds a significant layer of complexity to the immunogenicity of xenotransplanted pig islets.

ARE METHODS TO ALLEVIATE XENOGENEIC REJECTION MORE FEASIBLE OR PRACTICAL COMPARED TO THOSE USED FOR OTHER TRANSPLANT SOURCES?

Alleviating xenogeneic rejection involves several unique and sophisticated approaches due to the significant biological differences between species. These methods can be categorized into three main strategies: gene editing of the donor (e.g., pigs), pharmacological immunosuppression, and physical barriers such as encapsulation. Focusing specifically on gene modification efforts, pig islet xenotransplantation indeed represents the most extensive application of genetic engineering in the realm of islet transplantation (**Table 3**) [91]. The most extensively modified animals have undergone 69 genomic alterations, which include the removal of glycan antigens, the enhancement of human transgene expression, and the deactivation of porcine endogenous retroviruses [92]. This extensive level of genetic editing is primarily possible because of the wider array of modifications that are both ethically and technically feasible in pigs, in contrast to what is possible with human islets or stem cells. Conversely, evidence suggests that traditional immunosuppressive treatments, such as those based on tacrolimus, are less effective at managing the adaptive immune response against pig xenografts [93]. Instead, targeting the CD40/CD154 T-cell co-stimulation pathway has shown greater efficacy [94]. Notably, islets from adult wild-type (non-genetically modified) pigs have successfully functioned in diabetic non-human primates (NHPs) treated with anti-CD154 monoclonal antibody-based immunosuppression for up to 965 days [95]. Nonetheless, genetically editing pigs could potentially achieve comparable or superior outcomes with less aggressive immunosuppression (**Table 4**). The concern, however, lies in the intensity of immunosuppressive protocols required, particularly for conditions requiring long-term management like diabetes. Islet encapsulation may offer greater immediate benefits for pig islet transplantation compared to human islets [110]. This is due to pig islets' higher immunogenicity and the ample supply they offer, which is critical for overcoming xenotransplantation's unique challenges [111]. While both pig and human islet transplants can benefit from encapsulation technologies, the necessity and feasibility of these strategies might be more pronounced for pig islets to ensure successful transplantation outcomes. Although significant progress has been made [112–114], most strategies are yet to meet the criteria of obtaining sustainable and consistent diabetes management for >6 months in preclinical trials before they can be introduced in human clinical trials.

TABLE 3 | Targeted deletions/insertions in pig genome for xenotransplantation.

Gene modification	Purpose	Impact on transplantation
α1,3-Galactosyltransferase Knockout (GTKO) [69, 76]	Eliminates α-Gal epitopes to reduce hyperacute and acute vascular rejection	Significantly decreases antibody-mediated rejection; first major step towards viable xenotransplants. Knockout of the genes for the 3 glycan xenoantigens (providing triple-knockout, [TKO] pigs) is generally considered the basis of the pigs that will be sources of organs and cells for clinical transplantation
Cytidine monophosphate-N-acetylneuraminic acid hydroxylase Knockout (CMAH-KO) [77]	Eliminates Neu5Gc to reduce hyperacute and acute vascular rejection	
β-1,4N-acetylgalactosaminyltransferase Knockout (β4GalNT2-KO) [69]	Eliminates Sd(a) to reduce hyperacute and acute vascular rejection	
CD55 (DAF) Transgenic [78]	Regulates complement activation, reducing complement-mediated cell lysis	Enhances graft survival by protecting against complement-mediated damage
CD59 Transgenic [79]	Prevents the formation of the Membrane Attack Complex (MAC), protecting cells from complement-mediated lysis	Further protects xenografts from complement-mediated injury, complementing CD55 effects
CD46 (MCP) Transgenic [80]	Regulates complement activation on cell surfaces	Provides broad protection against complement activation, enhancing graft protection
Human Heme Oxygenase-1 (HO-1) Transgenic [81]	Provides cytoprotective, anti-inflammatory, and anti-apoptotic effects	Reduces ischemia/reperfusion injury and improves graft survival by mitigating acute inflammatory responses
Human Thrombomodulin (hTBM) Transgenic [82]	Modifies the coagulation cascade to reduce thrombosis in the graft	Addresses the issue of coagulation dysregulation in xenotransplants, improving graft function and longevity
α1,2-Fucosyltransferase (H Transferase) Transgenic [83]	Masks non-Gal antigens to further reduce antibody-mediated rejection	Complements GTKO by masking remaining xenoantigens, further reducing the immune response against the xenograft
SLA Class I and II Knockout [84, 85]	Reduces the expression of swine leukocyte antigens, decreasing T-cell mediated rejection	Aims to minimize direct T-cell recognition and response, lowering the risk of cellular rejection and the cross-reactivity of anti-HLA antibodies with SLA antigens
CD39 Transgenic [86]	Increases ATP and ADP hydrolysis, reducing platelet aggregation and thrombosis	Targets the prevention of thrombotic microangiopathy, promoting longer graft survival
PD-L1 Transgenic [87]	Inhibits T-cell activation and proliferation by engaging PD-1 on T cells	Contributes to creating an immunotolerant environment around the xenograft, reducing cellular rejection
HLA-E and HLA-G Transgenic [88, 89]	Engages inhibitory receptors on NK cells and certain T cells, reducing their activity	Aims to protect xenografts from NK cell-mediated damage and modulate T-cell responses, enhancing tolerance
CTLA4-Ig Transgenic [90]	Provides local immunosuppression by blocking costimulatory signals necessary for T-cell activation	Reduces the need for systemic immunosuppression, lowering side effects while protecting the graft

IS THERE EVIDENCE THAT PIG ISLETS ARE MORE EFFECTIVE IN REGULATING HUMAN BLOOD SUGAR LEVELS COMPARED TO OTHER SOURCES IN CLINICAL TRIALS?

Beyond the pioneering studies by Groth et al. [115] and Wang et al. [116], which examined free islet xenotransplantation, there's a notable scarcity of clinical trials in this area (**Table 5**). Instead, much of the focus has been on encapsulated islets transplanted without the need for immunosuppression [117]. These endeavors have yet to achieve unequivocal success. In several instances, improvements in blood sugar management could be attributed to rigorous medical oversight, including dietary changes, strict glucose monitoring, and specialized healthcare, rather than the transplanted pig islets themselves. However, research led by Matsumoto et al. stands out, having successfully reduced HbA1c levels significantly over 600 days in recipients of encapsulated pig islets, without resorting to immunosuppressive medications [118, 119]. Despite minimal side effects reported, the quest for more dependable and enhanced results continues. When these findings are weighed against the outcomes from human islet and stem cell-derived islet transplants, it's clear that transplants using human islets are currently the most effective for clinical management of diabetes, primarily due to their significant impact on glycemic control [6]. While pig islet xenotransplantation holds potential and has shown varied degrees of success, it still needs further development for it to be consistently reliable and widely applicable. On the other hand, islets derived from stem cells hold immense promise due to their potentially unlimited availability and fewer compatibility challenges. However, while their effectiveness has been shown in limited trials [120], confirmation of their clinical efficacy is still awaiting results from larger-scale studies.

IS THERE EVIDENCE TO SUGGEST THAT PORCINE ISLETS ARE SAFER AND MORE REGULATORILY STRAIGHTFORWARD COMPARED TO ALTERNATIVE SOURCES?

The safety and regulatory ease of using porcine islets compared to other sources such as human islets or stem cell-derived islets are areas of active research and debate. Adult porcine pancreatic islets could represent a safer and more effective alternative for therapeutic use than stem cells, due to their complete and immediate hormonal compatibility with human physiology

TABLE 4 | Immunosuppressive protocols associated with prolonged periods of insulin-independence and islet xenograft survival.

	Donor	Recipient	Islets (IEQ/kg)	Immunosuppression	Max graft survival (days)	Ref.
Wild type	Adult SNU Miniature Pig	STZ-induced diabetic rhesus monkeys	96,090	Anti-CD40 mAbs (2C10R4), Sirolimus, ATG, CVF, Tacrolimus, Adalimumab, Methylprednisolone	>320	[96]
			93,575	ATG, sirolimus, tacrolimus, anti-CD40 mAb, tocilizumab, CVF, adalimumab	176	[97]
			100,000	ATG, anti-CD154 mAbs (5C8), Sirolimus, CVF, TNF-a-neutralizing mAb (adalimumab)	603	[95]
			50,000–150,000	ATG, Rituximab, Belimumab, Sirolimus, Tacrolimus, Tofacitinib, Adalimumab, Anakinra, CVF, IVIg	201	[98]
			100,000	ATG, Belimumab, Sirolimus, Tacrolimus, Abatacept, Tofacitinib, Adalimumab, Anakinra, Tocilizumab, IVIg, Aspirin	222	[99]
			100,000	ATG, CVF, anti-CD154 mAbs (5C8), Anti-CD40 mAbs (2C10R4), Sirolimus, TNF-a-neutralizing mAb (adalimumab), Treg	965	[100]
			93,575	ATG, sirolimus, tacrolimus, anti-CD40 mAb, tocilizumab (IL-6 receptor antagonist), CVF, adalimumab	176	[97]
	Neonatal Duroc or Large White Crossbreeds	Rhesus macaques s/p pancreatectomy	50,000	Anti-CD 154 mAb, basiliximab, sirolimus, belatacept	>260	[101]
			50,000	Anti-CD40 mAbs (Chi220), aIL-2R (Basiliximab), Belatacept, Sirolimus	>203	[102]
		Rhesus macaques/STZ	50,000	MMF + CTLA4-Ig + LFA-3-Ig + anti-IL-2R + anti-LFA-1	114	[103]
	Adult Outbred swine and inbred miniature swine	Cynomolgus monkeys/STZ	25,000	Basilixumab, FTY720 or tacrolimus, everolimus, anti-CD154 mAb, leflunomide	>187	[104]
		Rhesus macaques/STZ	up to 280,000	Anti-ICAM-1 mAbs (MD-3), anti-CD154 mAbs (5C8), Sirolimus, TNF-a-neutralizing mAb (adalimumab), Anakinra, Ganciclovir, Clopidogrel, Heparin	520	[105]
Genetic Modifications	Adult GTKO hCD46 hCD39 or similar	Cynomolgus monkeys/STZ	85,000	ATG, Anti-CD154 mAbs (h5c8), MMF, Dextran sulfate, Prostacyclin, Methylprednisolone, Aspirin, Ganciclovir, Famotidine, Heparin	365	[22]
	Neonatal GTKO hCD55 hCD59 HT	Nondiabetic baboons	17,889	ATG, MMF, tacrolimus	30	[106]
	Adult GTKO	Cynomolgus monkeys/STZ	40,000	ATG, tacrolimus, rapamycin, anti-CD154 mAb, MMF	>58	[107]
	Adult, hCD46	Cynomolgus monkeys/STZ	85–100,000	ATG, Anti-CD154 mAb (ABI7953), Dextran sulfate, Methylprednisolone, Aspirin, Prostacyclin	396	[108]
	Fetal, hCD55	Cynomolgus monkeys	Not reported	Cyclosporine + steroids + cyclophosphamide or brequinar	7–40	[109]

and lower oncogenic risk, as they skip the need for differentiation and do not proliferate. While neonatal and fetal pig islets are less ideal due to their immature and proliferative characteristics, they likely still pose a lower oncogenic risk than stem cells. On the other hand, porcine pancreatic islets raise concerns regarding the risk of infectious disease transmission. Porcine islets carry the risk of transmitting porcine endogenous retroviruses (PERVs) to humans. However, extensive research and clinical trials have shown no evidence of PERV transmission so far, which is encouraging for their safety profile [121–125]. Nonetheless, the long-term risks of zoonotic disease transmission remain a concern that requires ongoing surveillance [126]. One of the key challenges limiting the widespread adoption of human islet allo-transplantation in countries like the USA is the stringent regulatory framework [127]. While porcine islets are subject to established xenotransplantation guidelines, which include rigorous safety and ethical evaluations, it is not entirely clear if they are better positioned to meet these requirements compared to human islets. Genetic modifications to reduce xenoreactivity and address safety concerns are promising but their effectiveness in fully satisfying regulatory criteria remains to be confirmed. Additionally, while regulatory frameworks for xenotransplantation aim to facilitate approval, the comparative ease with which porcine islets might navigate these requirements versus human islets is still uncertain. Ongoing research and regulatory developments will be essential to determining whether porcine islets can more effectively meet these stringent standards. Lastly, the employment of porcine islets in certain contexts could be perceived as more ethically permissible than the use of islets derived from stem cells, contingent upon the origins of these stem cells. Specifically, the ethical controversies surrounding the utilization of embryonic stem cells are not

TABLE 5 | Clinical trials involving porcine islets.

Trial	Islet source	Recipient details	Islet type	Average number of islets (IEQ/kg)	Site of transplant	Immunosuppression	Outcome
Groth et al. [115]	Swedish Landrace	T1D with kidney transplant (n = 10)	Fetal Free	200,000–1 million	Kidney capsule or intraportal	ATG, 15-deoxyspergualin, cyclosporine, prednisolone, azathioprine	No improvement in glycemic control
Elliott et al. [124, 137]	Cross-White Breed	T1D with and without kidney transplant (n = 1 each)	Neonatal Encapsulated	15,000	Peritoneum	None (n = 1) and Standard (n = 1)	Short-term insulin requirement and HbA1c improvement, no PERV transmission
Valdes-Gonzalez et al. [138, 139]	New Zealand bred	T1D adolescents (n = 12)	Neonatal with Sertoli cells	13,000–20,000 (first transplant)	Subcutaneous	None	Half achieved insulin reduction, improvement in HbA1c and less chronic complications
Wang et al. [116]	Xeno-1	T1D (n = 21)	Neonatal Free	55,000	Intraportal	Comprehensive regimen	Reduction in insulin requirements, improvement in HbA1c, no PERV transmission
Matsumoto et al. [118]	Auckland Island	T1D (n = 14)	Neonatal Encapsulated	5,000–20,000	Peritoneum	None	Reduction in unaware hypoglycemic events, minimal HbA1c or insulin dose change
Matsumoto et al. [119]	Auckland Island	T1D (n = 8)	Neonatal Encapsulated	5,000 and 10,000	Peritoneum	None	

relevant to porcine islets. Nonetheless, employing animal organs and tissues introduces distinct ethical challenges, such as those pertaining to the welfare of animals involved in breeding and procurement processes, as well as cultural and religious considerations. Regulatory agencies have established frameworks for xenotransplantation [128], which includes the transplantation of porcine islets. These frameworks address both safety and ethical issues, but they also mean that porcine islets must undergo rigorous preclinical and clinical testing to prove their safety and efficacy. In comparison, human islets and stem cell-derived islets, while also subject to strict regulatory scrutiny, are not confronted with the same level of concern regarding zoonotic disease transmission.

CONCLUSION

The quest for a cure for T1D through beta cell replacement therapy encompasses a dynamic interplay of potential and complexities. The efficacy of islet transplantation from human donors underscores the foundational promise of this approach by directly addressing T1D's root cause. However, the realization of a universal cure is hindered by several critical challenges: the quest for an inexhaustible and high-quality source of beta cells, the minimization or elimination of reliance on immunosuppressive drugs, and the assurance of the long-term viability and functionality of the transplanted cells. Alternative sources such as porcine islets and stem cell-derived islets offer intriguing possibilities, each with its unique set of advantages and challenges. Porcine islets, while providing a potentially unlimited supply, raise concerns regarding physiological compatibility, immunogenicity, and regulatory complexities. On the other hand, stem cell-derived islets, benefiting from advancements in cellular reprogramming and differentiation, appear to have a slight edge in current discussions, primarily due to their potential for unlimited supply and reduced ethical concerns compared to embryonic stem cells. However, the recent successes in whole organ xenotransplantation inject a renewed vigor into the exploration of porcine islets [129–135]. These advancements may pave the way for addressing some of the longstanding issues in a relatively short timeframe, particularly those related to immunogenicity and physiological compatibility [136]. This progress, alongside the ongoing refinement of techniques for stem cell-derived islets, underscores a dynamic research landscape. Thus, the future of beta cell replacement therapy for T1D remains an open field of scientific inquiry and innovation. It is propelled by the collective goal of developing a comprehensive, effective cure, navigating through the interwoven challenges of supply, compatibility, safety, and regulatory acceptance. The path forward is marked by the potential of recent breakthroughs and the promise of overcoming current limitations through concerted research efforts.

AUTHOR CONTRIBUTIONS

LP conceptualized the review, led the project, and reviewed the manuscript. AC analyzed literature and contributed to manuscript drafting and revisions. VT collected literature and assisted in drafting the manuscript. SP provided critical revisions focused on clinical insights. RC provided critical revisions focused on clinical insights and critically reviewed the manuscript. All authors approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Aspects of Regulation of Xenotransplantation in Europe

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The Council of Europe (CoE) and the European Union (EU) share the same fundamental values, i.e., human rights, democracy and the rule of law, but are separate entities which perform different, yet complementary, roles. The CoE brings together governments from across Europe, and beyond, to agree minimum legal standards in a wide range of areas. CoE monitors how countries apply the standards that they have chosen to sign up to. It provides technical assistance, often working together with the EU. The EU refers to those same European values as a key element of its political and economic integration processes. It often builds upon CoE standards when drawing up legal instruments and agreements which apply to the member states, furthermore, monitoring work in its dealings with neighbouring countries, many of which are CoE member states. At CoE, the European Committee on Organ Transplantation (CD-P-TO) is the steering committee in charge of organ transplantation activities. In the EU, the regulation on Substances of Human Origin (SoHO) was endorsed in 2024. The CoE and the EU thave concluded an agreement expanding their co-operation in the field of SoHO. In the BTC regulation, xenotransplantation is not included.

Keywords: regulation, Council of Europe, European Union, xenotransplantation, advanced therapy medicinal products

COUNCIL OF EUROPE

Founded in 1949, the CoE has 46 member states. The CoE is an international organization with the aim to protect human rights, democracy as well as the rule of law in all member states. The CoE cannot make binding laws, however it enforces international agreements reached by European states on various topics to achieve recommendations, conventions and treaties (https://www.coe.int/en/web/portal/european-union).

In 1999, the Committee of Ministers of the CoE set up a working party on XTx under the joint responsibility of the Steering Committee on Bioethics (CDBI) and the European Health Committee (CDSP) which decided to prepare a report on the state of the art in the field of XTx. The Report as well as an Explanatory Report to Recommendation Rec (2003)10 of the Committee of Ministers to member states on xenotranplantation were published in 2003 [1–3]. The report proposed that member states establish a mechanism for the registration and regulation of "certain aspects of XTx including (a) basic research and clinical trials, (b) the source and care of animals for use in XTx, (c) XTx programmes, (d) long term follow-up and review of XTx recipients and (e) the XTx source animals" [1]. The Recommendation Rec (2003)10 took "into account the shortage of organs and tissues of human origin available for transplantation" and it considered that "XTx might be one of the possible therapeutic responses to this shortage," further "noting that XTx remains largely an experimental activity and that research is essential for the achievement of progress in this field." Moreover, it stated the awareness "of the risks of rejection and illness XTx may cause in the recipient

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patient." Eventually, the recommendation was "mindful of the considerable risks which might arise from XTx in the field of public health and the transmission of diseases" [3].

The topic of XTx encompassing definitions and infectious risk is described briefly in the "Guide to the quality and safety of tissues and cells for human application" (T&C Guide); Chapter 5. Donor Evaluation, Subchapter 5.34 Relative Contraindications, f. Xenotransplantation; CD-P-TO, EDQM, 5th Edition, 2022 [4]. In the T&C Guide, XTx is defined as "any procedure that involves the transplantation, implantation or infusion into a human recipient of either (a) live cells, tissues, or organs from a nonhuman animal source, or (b) human body fluids, cells, tissues or organs that have had *ex vivo* contact with live nonhuman animal cells, tissues or organs. Biological products, drugs, or medical devices sourced from non-human animals that do not contain living cells, tissues or organs, including (but not limited to) porcine insulin, porcine heart valves, porcine skin and acellular porcine corneal stroma, and collagen matrices derived from acellular porcine, bovine or any other xenogeneic source are not considered as xenotransplantation" [4].

In 2023, the European Directorate for the Quality of Medicines & Healthcare (EDQM), European Committee (Partial Agreement) on Organ Transplantation (CD-P-TO) at the CoE accepted a proposal of member states to launch an update of the given report. The update was suggested due to the recent progress achieved in non-clinical (preclinical) and clinical activities in pig organ (heart, kidney) xenotransplantation to humans, particularly the xenotransplantations of genetically modified (gm) pig hearts to patients, performed as individual medical treatments in 2022 and 2023, Baltimore, United States, as well as recent activities in tissue and cellular xenotransplantation and extracorporeal exposure to xenoorgans, xenotissues and xenocells.

The state of the art of immunological and physiological barriers, non-clinical activities and of gm pigs as animal donors should be updated. Moreover, cultural, ethical and religious aspects of xenotransplantation, national policies as well as legislative and regulatory frameworks should be highlighted.

The updated report will not be placed neither as a legal instrument nor as a guide for transplant professionals, but rather as an overview of progress in the biomedicinal field which moves closer to the clinical arena. However, the update of the report may provide a basis for national legislation, European legislation as well as for international coordination and cooperation.

The timetable, designed for the years 2024–2026, is based on the formation of a working group formed by experts of the member states, the assignment of topics and chapters, drafting of an updated report and the consultation of the final report. At current, the working group elaborates an update of a questionnaire which was addressed to the member states in 1999 to compile the existing documents, laws and regulations within the CoE.

In view of the dynamics in the field of xenotransplantation, the World Health Organisation (WHO) that is part of the initiative suggested to implement other, non-European countries and societies in the project, such as The Transplantation Society (TTS) and the International Xenotransplantation Association (IXA).

EUROPEAN UNION

In the European Commission Directive 2006/17/EC (§ 1.1.13 of Annex I) "transplantation with xenografts" is included as general criteria for exclusion of deceased human donors of tissues and cells, related to the fact that exposure of human recipients to non-human live animal material has the potential for cross-species infection, caused by infectious agents such as porcine endogenous retroviruses (PERV) that are integrated into the pig genome [5, 6] and other exogenous infectious agents, e.g., porcine cytomegalovirus (PCMV) [7].

The EU comprises 27 member states. In the EU, a regulatory framework for XTx is based on guidelines and ordinances on advanced therapy medicinal products (ATMP) [8], pharmacovigilance and clinical trials.

The fundamental rights of both animals as donors and humans as recipients of organs, tissues, and cells are adequately protected by the framework.

Moreover, in the EU member states, national laws may be implemented, such as those on genetic engineering, protection against infection and medicinal products, e.g., the German Medicinal Products Act.

The following definitions shall apply for the purposes of the ATMP regulation (EC) No 1394/2007 [8]:

"(a) 'Advanced therapy medicinal product' means any of the following medicinal products for human use: (1) a gene therapy medicinal product as defined in Part IV of Annex I to Directive 2001/83/EC, (2) a somatic cell therapy medicinal product as defined in Part IV of Annex I to Directive 2001/83/EC, (3) a tissue engineered product as defined in point (b).

(b) 'tissue engineered product' means a product that (1) contains or consists of engineered cells or tissues, and (2) is presented as having properties for, or is used in or administered to human beings with a view to regenerating, repairing or replacing a human tissue.

A tissue engineered product may contain cells or tissues of human or animal origin, or both. The cells or tissues may be viable or non-viable. It may also contain additional substances, such as cellular products, bio-molecules, bio-materials, chemical substances, scaffolds or matrices.

Products containing or consisting exclusively of non-viable human or animal cells and/or tissues, which do not contain any viable cells or tissues and which do not act principally by pharmacological, immunological or metabolic action, shall be excluded from this definition.

(c) Cells or tissues shall be considered 'engineered' if they fulfil at least one of the following conditions: the cells or tissues have been subject to substantial manipulation, so that biological characteristics, physiological functions or structural properties relevant for the intended regeneration, repair or replacement are achieved. The manipulations listed in Annex I, in particular, shall not be considered as substantial manipulations."

For XTx, the ATMP regulation displays some limitations since animal organs are not explicitly mentioned, even though they may be derived from genetically modified (gm) animals. As a consequence, those animal organs would be substantially manipulated compared with organs that are harvested from wild-type animals.

In the ATMP regulation, the definition of somatic cell therapeutics, as well as the definition of tissue-engineered products of animal origin, is based on tissues or cells; however, it excludes organs. Naturally, organs derived from gm animals contain tissues and cells.

To this end, the European Medicines Agency (EMA, Amsterdam, Netherlands) has published the guideline on xenogeneic cell-based medicinal products [9]. "This guideline addresses the scientific requirements for xenogeneic cell-based medicinal products for human use. Xenogeneic cell-based medicinal products contain viable animal cells or tissues as the active substance. Xenogeneic materials might be sourced either from non-transgenic or transgenic animals. The animal cells can also be genetically modified. Although not within the scope of the guidance, some of general principles of this guideline will apply to viable animal cells used as raw materials (e.g., feeder cells) and/or where contamination with xenogeneic material is possible. This guideline is intended for products entering the marketing authorisation (MA) procedure. However, the principles laid down in the guideline should be considered by applicants entering into clinical trials." The legal basis for the guideline should be read in conjunction with ATMP regulation (EC) No 1394/2007 [8].

Central elements of the ATMP regulation include (a) designation of the EMA to grant marketing authorizations for XTx products within the EU, (b) requirement for traceability of xenogeneic organs, tissues and cells, from creation through clinical use and ultimate disposition, and (c) hospital exemption for medicinal products that are not routinely prepared.

In the EU, regulatory pathways to yield marketing authorizations for medicinal products, including ATMP, are based on data that cover product quality, nonclinical assessment (i.e., preclinical trials), as well as clinical trials.

Data must be summarized by the applicant, often the pharmaceutical entrepreneur working in partnership with clinical investigators and their medical institution(s), in dossiers including an internationally standardized set of Common Technical Documents (CTD). The application is evaluated by the European National Competent Authorities (NCA; in Germany the Paul-Ehrlich-Institut, Federal Institute for Vaccines and Biomedicines, Langen) that are nominated as rapporteur and co-rapporteur by EMA. The CTD are expected to show consistent data on the quality, safety, and efficacy of the particular product.

Beforehand, EMA and NCA offer central and national scientific recommendations on the classification of ATMP.

To ensure that research, development, and regulation remain current, the regulatory framework in the EU and its member states will be adjusted to appropiately reflect scientific and technical advancements in xenotransplantation.

In the EU, the regulation on Substances of Human Origin (SoHO) covering blood, tissues and cells (BTC), except solid organs, was endorsed in 2024. The CoE/EDQM and the EU, through the European Commission, have concluded an agreement expanding the scope of their co-operation in the field of SoHO[1]. The field of XTx is not part of the BTC regulation.

WORLD HEALTH ORGANISATION

The WHO has a long-standing interest in xenotransplantation, which started with the publication of the "WHO Guidance on Xenogeneic Infection/Disease Surveillance and Response: A strategy for International Cooperation and Coordination" in 2001 [10].

The next step was resolution WHA57.18 of the 57th World Health Assembly in 2004, urging member states, amongst others, to perform xenogeneic transplantation only "when effective national regulatory control and surveillance mechanisms overseen by national health authorities are in place" [11].

This resolution was preceded by a position report on ethical aspects published by the IXA pointing to the requirements of adequate preclinical data, proper oversight by competent authorities, and approval by institutional bodies over-seeing the ethical conduct of human research and animal welfare [12].

Resolution 57.18 led to four WHO-supported global consultations. The first Xenotransplantation Advisory Consultation was held in Geneva, Switzerland, in 2005 [13].

It was followed by the First WHO Global Consultation on Regulatory Requirements for Xenotransplantation Clinical Trials, which was organized in collaboration with the Ministry of Health of China, the International Xenotransplantation Association and the University of Central-South China in Changsha, China, in 2008. The recommendations were published as the "Changsha Communiqué" [14] to develop and to update regulatory requirements for xenotransplantation clinical trials.

The Second WHO Global Consultation on Regulatory Requirements for Xenotransplantation Clinical Trials was organized in collaboration with the TTS and the IXA in Geneva, Switzerland, in 2011, and had a focus on xenotransplantation-associated infectious risk [15, 16].

The Third WHO Global Consultation on Regulatory Requirements for Xenotransplantation Clinical Trials was organized in collaboration with the IXA and the EMA in Changsha, China, December 2018 [17]. The principles and recommendations of the "Changsha Communiqué" were reviewed and discussed in detail by different working parties charged with covering the following topics: (a) xenozoonosis; (b) regulatory; (c) biorepository; (d) transgenic pig facilities; (e) biomaterials and encapsulation; and (f) immunosuppression and tolerance induction. The guidance document from the

[1]https://www.edqm.eu/en/-/the-council-of-europe/edqm-and-the-european-union-conclude-an-agreement-expanding-the-scope-of-their-co-operation-in-the-field-of-substances-of-human-origin

Second Global Consultation (Geneva, 2011) was included in these discussions.

Eventually, the consultation focused on drafting proposed revisions of the WHO documents, and resulted in the formulation of the draft "Third WHO Global Consultation on Regulatory Requirements for Xenotransplantation Clinical Trials, The 2018 Changsha Communiqué" [17].

The draft was submitted to WHO in February 2019 for WHO and World Health Assembly consideration where it has not been finalized yet.

CONCLUSION

XTx is separately and partially covered by the CoE, the EU, as well as the World Health Organisation (WHO). So far, there is indirect interaction and partial linkage between the COE, the EU and the WHO.

AUTHOR CONTRIBUTIONS

RT as the only author is responsible for the presentation of the state of the art of regulation of xenotransplantation in Europe including the European Union.

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CONFLICT OF INTEREST

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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