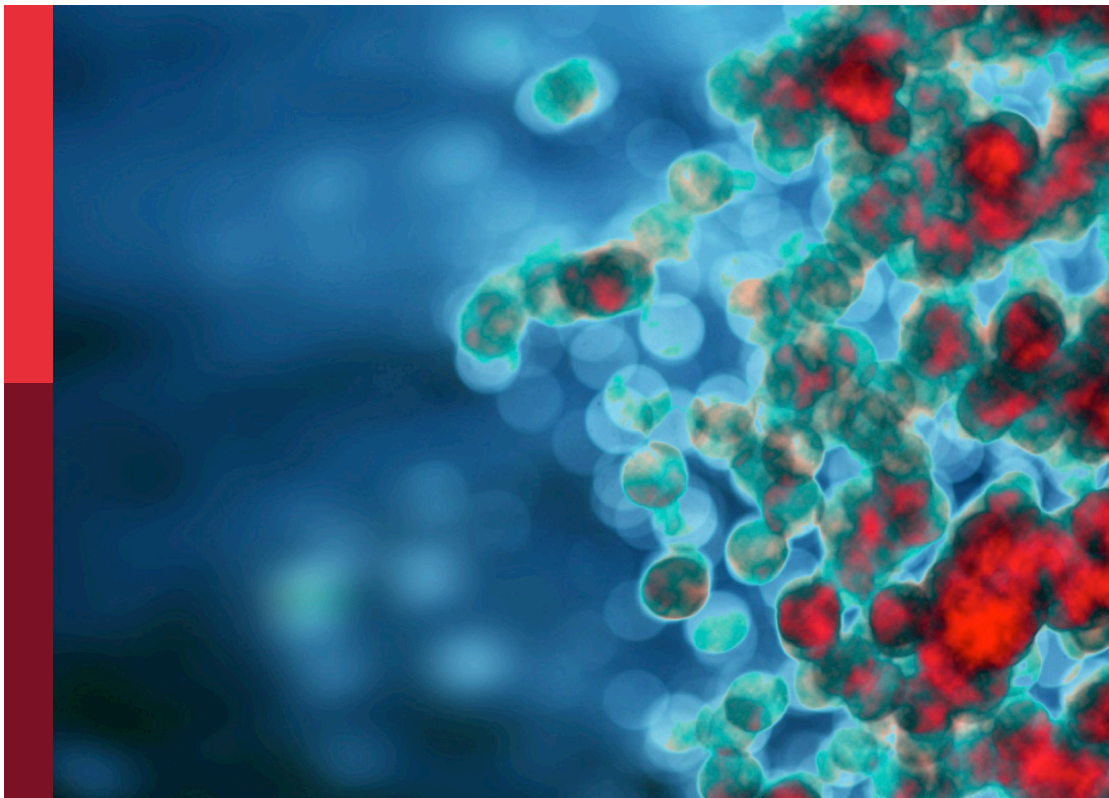
frontiers | Research Topics

Community series in progress of allo- and xeno-transplantation facilitating the initial xeno-kidney and islet clinical trials, volume II

Edited by
Lisha Mou and Burcin Ekser

Published in
Frontiers in Immunology



ISSN 1664-8714
ISBN 978-2-8325-5065-6
DOI 10.3389/978-2-8325-5065-6

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Community series in progress of allo- and xeno-transplantation facilitating the initial xeno-kidney and islet clinical trials, volume II

Topic editors

Lisha Mou — Shenzhen Second People's Hospital, China

Burcin Ekser — Indiana University Bloomington, United States

Citation

Mou, L., Ekser, B., eds. (2024). *Community series in progress of allo- and xeno-transplantation facilitating the initial xeno-kidney and islet clinical trials, volume II*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-5065-6

Table of contents

frontiers | Frontiers in Immunology

TYPE Editorial
PUBLISHED 13 June 2024
DOI 10.3389/fimmu.2024.1439832

OPEN ACCESS

EDITED AND REVIEWED BY
Antoine Toubert,
Université Paris Cité, France

*CORRESPONDENCE
Lisha Mou
lishamou@email.szu.edu.cn

RECEIVED 28 May 2024
ACCEPTED 05 June 2024
PUBLISHED 13 June 2024

CITATION
Mou L and Ekser B (2024) Editorial: Community series in progress of allo- and xeno-transplantation facilitating the initial xeno-kidney and islet clinical trials, volume II. *Front. Immunol.* 15:1439832. doi: 10.3389/fimmu.2024.1439832

Editorial: Community series in progress of allo- and xeno-transplantation facilitating the initial xeno-kidney and islet clinical trials, volume II

Lisha Mou[1,2*] and Burcin Ekser[3]

[1]Department of Endocrinology, Institute of Translational Medicine, The First Affiliated Hospital of Shenzhen University, Shenzhen Second People's Hospital, Shenzhen, China, [2]MetaLife Lab, Shenzhen Institute of Translational Medicine, Health Science Center, The First Affiliated Hospital of Shenzhen University, Shenzhen Second People's Hospital, Shenzhen, China, [3]Division of Transplant Surgery, Department of Surgery, Indiana University School of Medicine, Indianapolis, IN, United States

KEYWORDS

xenotransplantation, islet transplantation, kidney transplantation, genetically modified porcine endothelial cells, PD-L1 overexpression, genetic modifications in pigs, microenvironment in transplantation, hepatocyte microencapsulation

Editorial on the Research Topic
Community series in progress of allo- and xeno-transplantation facilitating the initial xeno-kidney and islet clinical trials, volume II

The advent of xenotransplantation has ushered in a new era of possibilities in addressing the severe organ shortage crisis. The current Research Topic in this volume of "Progress of Allo- and Xeno-transplantation Facilitating the Initial Xeno-Kidney and Islet Clinical Trials" offers a collection of pioneering studies that explore various facets of xenotransplantation, particularly focusing on kidney and islet transplantation. This editorial provides an overview of the contributing articles, highlighting their key findings and placing them within the broader context of transplantation research.

1 Challenges and opportunities in the islet transplantation microenvironment

Chen et al. presented a comprehensive summary of the challenges and opportunities in the islet transplantation microenvironment. Their study underscores the importance of the microenvironment in determining transplantation outcomes and offers insights into potential strategies to improve islet graft survival with a focus on inflammatory cytokines, immune cells, and vascular endothelial cells.

2 Pancreatic islet transplantation: current advances and challenges

In a similar topic but with a different approach, Wang et al. provided a thorough review of the current advances and challenges in pancreatic islet transplantation. They discuss the issues related to islet sourcing, transplantation sites, and immune rejection. Their study highlights the feasibility of inducing stem cells to differentiate into β-like cells *in vitro* and explores the potential of porcine islets in addressing the shortage of islet donors.

3 Co-expression of HLA-E and HLA-G on genetically modified porcine endothelial cells

To improve the outcomes in xenotransplantation, Cross-Najafi et al. investigated the co-expression of HLA-E and HLA-G on genetically modified porcine endothelial cells. Their findings demonstrated that the co-expression of HLA-E and HLA-G can significantly attenuate human NK cell-mediated degranulation, shedding light on more successful xenotransplantation outcomes.

4 Combined islet and kidney xenotransplantation for diabetic nephropathy

A mini-review by Eisenson et al. provided an update on the ongoing research in combined islet and kidney xenotransplantation for diabetic nephropathy. The authors highlighted the potential of this dual approach in addressing both diabetes and kidney failure, showcasing promising preliminary results with evidence from the published literature.

5 Human PD-L1 overexpression in porcine kidneys

In original research by Schmalkuche et al., the effect of human PD-L1 overexpression in porcine kidneys has been explored. This interesting study showed that human PD-L1 genetic modification can reduce xenogeneic human T-cell immune responses, thus enhancing the viability of porcine kidneys in case of clinical xenotransplantation.

6 Genetically modified pigs targeting complement activation

Sun et al. discussed the cutting-edge genetic modifications in pigs aimed at targeting complement activation, which has been a major barrier in xenotransplantation. Their study provides valuable insights into the genetic engineering techniques that can mitigate immune rejection.

7 Microenvironment and survival in kidney transplantation

Huang et al. conducted a bibliometric analysis to examine the relationship between the microenvironment and survival in kidney transplantation. Their analysis identifies key trends and research hotspots, offering a roadmap for future studies in this critical area.

8 Advances in hepatocyte microencapsulation

Wang et al. reviewed the advances in hepatocyte microencapsulation, focusing on selecting materials and preservation methods. Their comprehensive review highlights the progress made in enhancing the viability and functionality of encapsulated hepatocytes for transplantation.

9 Developments in kidney xenotransplantation

Xu and He presented a detailed overview of the developments in kidney xenotransplantation. Their study highlights the significant strides made in genetic modifications and immunosuppressive protocols, which are crucial for the success of clinical xenotransplantation.

10 Ethical and legislative advances in xenotransplantation

With his expertise over decades, Hawthorne discussed the ethical and legislative advances in xenotransplantation, with a main focus on cardiac xenotransplants. His paper emphasizes the importance of ethical considerations and regulatory frameworks in advancing xenotransplantation to clinical practice in a safe manner.

11 Anesthesia and surgery in kidney xenotransplantation

Zhang et al. explored the role of anesthesia and surgical techniques in advancing kidney xenotransplantation to clinical practice. Their study bridges the gap between preclinical and clinical practices, offering insights into optimizing surgical outcomes.

The collection of articles by many experts in this volume provides a comprehensive overview of the current state of xenotransplantation research. Each study contributes valuable

knowledge to the field, addressing various challenges and proposing innovative solutions.

As we move closer to clinical reality with limited cases happening all over the world, it is imperative to continue interdisciplinary collaboration and rigorous research to overcome the remaining hurdles in xenotransplantation worldwide. This collective effort will lead to successful clinical trials and ultimately, a wider application of xenotransplantation in addressing the organ shortage crisis globally.

Author contributions

LM: Writing – original draft. BE: Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was supported by the Shenzhen Science and Technology Program (JCYJ20230807115107015, GCZX2015043017281705), the National Key R&D Program of China (2017YFC1103704), Shenzhen High-level Hospital Construction Fund (2019).

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.

Publisher's note

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 17 July 2023
DOI 10.3389/fimmu.2023.1217809

OPEN ACCESS

EDITED BY
Sina Naserian,
Hôpital Paul Brousse, France

REVIEWED BY
Eva Maria Weissinger,
Hannover Medical School, Germany
Mohamed Essameldin Abdelgawad,
Wake Forest University, United States

*CORRESPONDENCE
Ping Li
pili@iu.edu
Burcin Ekser
bekser@iupui.edu

RECEIVED 05 May 2023
ACCEPTED 30 June 2023
PUBLISHED 17 July 2023

CITATION
Cross-Najafi AA, Farag K, Isidan A, Li W, Zhang W, Lin Z, Walsh JR, Lopez K, Park Y, Higgins NG, Cooper DKC, Ekser B and Li P (2023) Co-expression of HLA-E and HLA-G on genetically modified porcine endothelial cells attenuates human NK cell-mediated degranulation. *Front. Immunol.* 14:1217809. doi: 10.3389/fimmu.2023.1217809

Co-expression of HLA-E and HLA-G on genetically modified porcine endothelial cells attenuates human NK cell-mediated degranulation

Arthur A. Cross-Najafi[1], Kristine Farag[1], Abdulkadir Isidan[1], Wei Li[2], Wenjun Zhang[1], Zhansong Lin[3], Julia R. Walsh[1], Kevin Lopez[1], Yujin Park[1], Nancy G. Higgins[4], David K.C. Cooper[5], Burcin Ekser[1]* and Ping Li[1]*

[1]Transplant Division, Department of Surgery, Indiana University School of Medicine, Indianapolis, IN, United States, [2]Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN, United States, [3]Ragon Institute of Massachusetts General Hospital (MGH), Massachusetts Institute of Technology (MIT), and Harvard, Cambridge, MA, United States, [4]Transplant Immunology, Indiana University Health, Indianapolis, IN, United States, [5]Center for Transplantation Sciences, Massachusetts General Hospital/Harvard Medical School, Cambridge, MA, United States

Natural killer (NK) cells play an important role in immune rejection in solid organ transplantation. To mitigate human NK cell activation in xenotransplantation, introducing inhibitory ligands on xenografts *via* genetic engineering of pigs may protect the graft from human NK cell-mediated cytotoxicity and ultimately improve xenograft survival. In this study, non-classical HLA class I molecules HLA-E and HLA-G were introduced in an immortalized porcine liver endothelial cell line with disruption of five genes (*GGTA1*, *CMAH*, *β4galNT2*, *SLA-I α chain*, and *β-2 microglobulin*) encoding three major carbohydrate xenoantigens (αGal, Neu5Gc, and Sda) and swine leukocyte antigen class I (SLA-I) molecules. Expression of HLA-E and/or HLA-G on pig cells were confirmed by flow cytometry. Endogenous HLA-G molecules as well as exogenous HLA-G VL9 peptide could dramatically enhance HLA-E expression on transfected pig cells. We found that co-expression of HLA-E and HLA-G on porcine cells led to a significant reduction in human NK cell activation compared to the cells expressing HLA-E or HLA-G alone and the parental cell line. NK cell activation was assessed by analysis of CD107a expression in $CD3^{-}CD56^{+}$ population gated from human peripheral blood mononuclear cells. CD107a is a sensitive marker of NK cell activation and correlates with NK cell degranulation and cytotoxicity. HLA-E and/or HLA-G on pig cells did not show reactivity to human sera IgG and IgM antibodies. This *in vitro* study demonstrated that co-expression of HLA-E and HLA-G on genetically modified porcine endothelial cells provided a superior inhibition in human xenoreactive NK cells, which may guide further genetic engineering of pigs to prevent human NK cell mediated rejection.

KEYWORDS

xenotransplantation, natural killer cells, immune tolerance, immune rejection, HLA-E, HLA-G, inhibitory ligands and receptors, degranulation

Introduction

Pig-to-human xenotransplantation offers a promising solution to address the persistent organ shortage (1). Interspecies incompatibilities result in robust human immune responses directed against the porcine xenograft. The consequence is rapid destruction and failure of the transplanted organ. Genetic modification (GM) of pigs has proven to be a valuable strategy for improving pig-human compatibility (2). Recent advancements in the genetic engineering of pigs have brought us closer to achieving successful xenotransplantation (3). In 2022, the first genetically modified pig-to-human cardiac xenotransplant was performed, which kept the recipient alive for two months (4). This groundbreaking event marks an important turning point: hyperacute xenograft rejection is no longer an absolute contraindication to xenotransplantation. Despite this exciting fact, acute and chronic organ rejection remain major barriers to successful pig-to-human xenotransplantation. To achieve long-term survival of pig xenografts and reduce the need for life-long immunosuppressive therapy with deleterious side effects, further GMs of pig tissues and organs are needed. These GMs will aim to reduce cell-mediated immune responses and improve major histocompatibility complex (MHC) compatibilities (5–10).

Human NK cells comprise the first line of defense of the innate immune system and are also involved in adaptive immunity. In solid organ transplantation, NK cell infiltration has been characterized with increased graft rejection in both allografts and xenografts (11, 12). NK cells can discriminate self, non-self, and abnormal cells (virus-infected cells or tumor cells) quickly, using a variety of cell-surface receptors which interact with the ligands on target cells (13). The balance of inhibitory and activating signals determines NK cell activation or inhibition. NK cell inhibitory ligands such as non-classical human leukocyte antigens (HLA)-E and -G are highly expressed in the human placenta (14), and contribute to establishing and maintaining immune tolerance at the maternal-fetal interface (15). Attempts have been made to investigate the role of HLA-E and HLA-G on porcine cells in regulating human NK cell activation *in vitro* and different inhibition pathways have been revealed (16, 17). Unlike classical HLA class I molecules, HLA-E and HLA-G display a limited polymorphism and are not considered in HLA typing for allotransplantation (18, 19). HLA-G plays an immunomodulatory role by binding the inhibitory receptors: Ig-like transcript 2 (ILT2) on dendritic cells, B cells, NK cells, and T cells; ILT4 on cells of myeloid origin; and killer cell immunoglobulin-like receptor 2DL4 (KIR2DL4) on NK cells (20–23). HLA-G expression is beneficial and promotes graft tolerance in solid organ transplantation, as evidenced by increased HLA-G expression in allografts and/or plasma correlating with improved graft acceptance (24, 25). Forte et al. reported that HLA-G expression inhibits the rolling adhesion of activated human NK cells on porcine endothelial cells (26) and partially protects porcine cells against direct human NK cytotoxicity (27). The protective role of HLA-E on porcine cells in human NK cell-mediated cytotoxicity has been reported (10, 28, 29). The HLA-E molecules present a highly conserved set of nonameric peptides (VL9) derived from the leader sequence of HLA-A/B/C/G molecules to NK cells and specific CD8 T cells (30). HLA-E-VL9 complex is a major inhibitory ligand for the NK inhibitory receptor NKG2A (31). VL9 peptides stabilize HLA-E molecules and determine HLA-E expression on cell surface. Previous studies demonstrated that HLA-E molecule loaded with the HLA-G leader peptide exhibited the highest affinity for NKG2A receptor (32) and co-expression HLA-G and HLA-E on swine endothelial cells efficiently enhanced the inhibition of NK cell-mediated cytotoxicity (33). Recent *ex vivo* studies indicated that transgenic expression of HLA-E attenuated porcine lung xenograft injury and reduced NK cell recruitment in pig limbs when perfused with human blood (34, 35).

In this study, an immortalized porcine liver-derived endothelial cell line (ipLDEC) with five-gene knockout (5GKO) (36) was used to express HLA-E, HLA-G, or co-express HLA-E and HLA-G molecules, namely 5GKO.HLA-E, 5GKO.HLA-G, and 5GKO.HLA-E.HLA-G cells. Human NK cell responses to these three modified cells as well as the parental 5GKO cells were evaluated by examining CD107a surface expression on $CD3^-$ $CD56^+$ population. CD107a, also known as lysosomal-associated membrane protein-1 (LAMP-1), is a functional marker for NK cell activation, which correlates with both cytokine secretion and NK cell-mediated cytotoxicity (37). The reactivity of human antibodies to HLA-E and/or HLA-G-expressing porcine cells was examined by a flow cytometry-based assay.

Materials and methods

Establishment of genetically modified porcine endothelial cell lines

The five-gene knockout cell line (5GKO, *GGTA1/CMAH/β4galNT2/SLA-I α chain/β-2 microglobulin*) was generated from ipLDEC, as previously described (36). The 5GKO cell line served as the parental cell line to express HLA-E and/or HLA-G molecules.

HLA-G is a heterodimer protein consisting of a heavy chain and β-2 microglobulin (B2M) subunits encoded by two genes located on different chromosomes. A single chain gene was designed by linking the *HLA-G heavy chain* (NCBI reference number: NM_001363567.2) and *B2M* (NCBI reference number: NM_004048.4) genes with self-cleaving peptide P2A DNA fragment, synthesized by Integrated DNA Technologies (IDT, Coralville, IA), and inserted downstream of the CMV promoter in an expression vector derived from pEGFP-N1, which had *EGFP* gene removed (Figure 1A). This recombinant plasmid was delivered into 5GKO ipLDEC by electroporation using the Neon Transfection System (Thermo Fisher Scientific, Waltham, MA). The transfected cells were cultured in selective media containing G418 at 200 ng/mL for 10 days. HLA-G expression was verified by flow cytometry using PE-conjugated mouse anti-HLA-G antibody (Clone 87G, BioLegend, San Diego, CA). 5GKO cells were used as a control (Figure 1B). 5GKO.HLA-G cells were isolated by a BD FACSAria Fusion cell sorter (BD Biosciences, San Jose, CA) (Figure 1C).

The HLA-E molecule is a trimeric complex, consisting of a heavy chain, B2M, and a signal peptide derived from other HLA class I molecules (30). To ensure HLA-E expression in porcine cells,

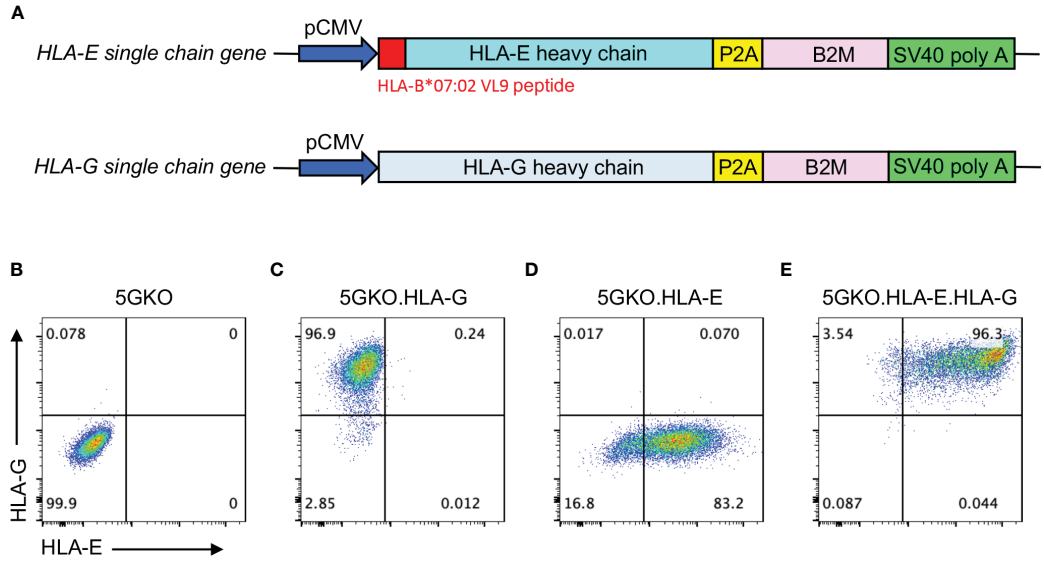


FIGURE 1
Expression of HLA-E and/or HLA-G on 5GKO cells. **(A)** Schematic of expression cassettes with a single chain gene of HLA-E and HLA-G. Flow cytometry analysis of cell surface expression of HLA-E and HLA-G on 5GKO **(B)**, 5GKO.HLA-G **(C)**, 5GKO.HLA-E **(D)**, and 5GKO.HLA-E.HLA-G **(E)** cell lines.

the *HLA-E heavy chain* gene (HLA-E*010301 allele, NCBI reference number: NM_005516.6) was modified by replacing its original signal peptide DNA sequence with *HLA-B*07:02* signal peptide (VMAPRTVLL, NCBI Reference Sequence: NM_005514.8) DNA sequence, then linked to *B2M* gene with P2A DNA fragment. This single-chain *HLA-E* gene was synthesized by IDT and subsequently cloned to the downstream of the CMV promoter in an expression vector derived from pEGFP-N1 (Figure 1A). This plasmid was delivered into 5GKO and 5GKO.HLA-G cells by electroporation, respectively. The transfected cells were cultured in selective media containing G418 at 200 ng/mL for 10 days. HLA-E expression was confirmed by flow cytometry using APC-conjugated mouse anti-HLA-E antibody (Clone 3D12, BioLegend). 5GKO.HLA-E and 5GKO.HLA-E.HLA-G cells were isolated by a BD FACSAria Fusion cell sorter (BD Biosciences) using APC-conjugated mouse anti-HLA-E antibody and PE-conjugated mouse anti-HLA-G antibody (BioLegend) (Figures 1D, E). Both HLA-E antibody and HLA-G antibody are specific. Cross-reactivity of HLA-E antibody to HLA-G molecules or HLA-G antibody to HLA-E molecules has not been observed.

Stability and expression level of HLA-E and HLA-G on 5GKO cell lines

HLA-E and HLA-G surface expression on 5GKO cells were examined four times for three weeks after flow sorting. 5GKO.HLA-E, 5GKO.HLA-G, and 5GKO.HLA-E.HLA-G were stained with APC-conjugated mouse anti-HLA-E antibody and PE-conjugated mouse anti-HLA-G antibody (BioLegend), as described above. HLA-E and HLA-G expression were measured by the percentage of positive cells as well as the mean fluorescence of intensity (MFI). The percentage of HLA-E or HLA-G positive cells was compared between 5GKO.HLA-E and 5GKO.HLA-E.HLA-G cells or between 5GKO.HLA-G and 5GKO.HLA-E.HLA-G cells at each time point. HLA-E MFI was compared between 5GKO.HLA-E and 5GKO.HLA-E.HLA-G cells while HLA-G MFI was compared between 5GKO.HLA-G and 5GKO.HLA-E.HLA-G cells.

HLA-E expression on 5GKO.HLA-E cells pulsed with HLA-G VL9 peptides

HLA-G VL9 peptide (VMAPRTLFL) was synthesized at purity of 95.1% by GenScript Biotech (Piscataway, NJ). 5GKO.HLA-E cells were incubated with HLA-G peptides at final concentrations of 25 μM, 50 μM, and 100 μM in a CO_2 incubator at 37°C overnight. 5GKO.HLA-E cells alone were used as a control. HLA-E expression on pig cells was measured by APC-conjugated mouse anti-HLA-E antibody staining and analyzed by an LSRFortessa flow cytometer (BD Biosciences). Experiments were repeated three times with similar results.

Human NK cell degranulation in response to porcine endothelial cell stimulation

NK cell degranulation assay was performed in a similar fashion as previously described (36, 38). CD107a is a functional marker and widely used for identification of NK cell activity (37, 39, 40). Commercially available buffy coats were acquired from Versiti Indiana Blood Center. Fresh whole blood was drawn from two human donors following the guidelines of the Institutional Review Board (IRB) of Indiana University, IRB#11013. Human peripheral

blood mononuclear cells (PBMCs) were isolated from buffy coat and fresh whole blood using Ficoll-Paque Plus (GE-Healthcare, Pittsburgh, PA) and Lymphoprep (STEMCELL Technologies, Vancouver, Canada) gradient centrifugation, respectively, for a total of 5 human donors. PBMCs from 5 donors were cultured in RPMI1640 with 10% FBS, 1% penicillin/streptomycin, and 20 ng/mL recombinant human IL-2 (rhIL-2) (BioLegend) at 37°C in a 5% CO_2 incubator for 5 days. 5GKO, 5GKO.HLA-E, 5GKO.HLA-G, and 5GKO.HLA-E.HLA-G cells were plated at 5 $\times 10^4$ per well in a Biocoat collagen I-coated 48-well plate (Corning Incorporated, Corning, NY) one day prior to co-culture. PBMCs were added to porcine cells at 5 $\times 10^5$ per well and co-cultured for 2 hours at 37°C in a CO_2 incubator. Cultured cells were then collected and stained with fixable viability dye eFluor 780 (Thermo Fisher Scientific) and fluorochrome-conjugated antibodies against human CD45, CD3, CD56, and CD107a (BioLegend). Cells were fixed with 2% PFA for 15 minutes at room temperature and subsequently analyzed using an LSRFortessa flow cytometer (BD Biosciences). 70,000 - 80,000 events were acquired in lymphocyte gate. After pre-gating on $CD45^+$ live singlets, NK cell degranulation activity was assessed by the percentage of CD107a positive cells in a $CD3^-CD56^+$ cell population. Flow data were analyzed using FlowJo v10 software (BD Biosciences). The experiment was repeated three times to obtain technical replicates.

Human serum antibody reactivity to porcine endothelial cells expressing HLA-E and HLA-G

Human antibody binding to porcine endothelial cells was examined, as previously described (36). Briefly, 2×10^5 porcine cells (5GKO, 5GKO.HLA-E, 5GKO.HLA-G, and 5GKO.HLA-E.HLA-G) were washed and incubated with 25% heat-inactivated human serum in EX-CELL 610-HSF serum-free medium (Sigma, St. Louis, MO) with 0.1% sodium azide for 1 hour at 4°C. Human sera were obtained from patients on the kidney transplant waitlist, 10 sera with high panel reactive antibody (PRA) and 10 sera with low PRA, for a total of 20 samples (n = 20). Each pig cell line was washed three times with EX-CELL 610-HSF serum-free medium and stained with goat anti-human IgG Alexa Fluor 488 or donkey anti-human IgM Alexa Fluor 647 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 30 minutes at 4°C, respectively. Cells were washed, fixed with 2% PFA for 15 minutes at room temperature, and subsequently analyzed using an LSRFortessa flow cytometer (BD Biosciences). Flow data were analyzed using FlowJo v10 software (BD Biosciences). Each pig cell line stained with goat anti-human IgG Alexa Fluor 488 or donkey anti-human IgM Alexa Fluor 647 was used as background and subtracted from each corresponding sera binding group. Human IgG and IgM bindings to pig cells were analyzed by stratification into low PRA and high PRA sera groups. The difference between low PRA and high PRA sera binding to each individual modified cell line was also compared.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software, San Diego, CA). A normality test was used to assess data distribution. An ordinary one-way ANOVA multiple comparisons test with Šídák's correction was used to analyze the differences among multiple groups. Student's t-test was used to analyze the differences between the two groups. A p-value less than 0.05 was considered statistically significant.

Results

Expression of HLA-E and/or HLA-G molecules on porcine endothelial cells

Three porcine cell lines expressing HLA-E, HLA-G, and co-expressing HLA-E and HLA-G were successfully established (Figures 1C–E). Stability of HLA-E and HLA-G molecules on porcine cells was examined. During the three-week culture, HLA-E expression on 5GKO.HLA-E.HLA-G was much more stable compared to HLA-E on 5GKO.HLA-E cells as indicated by the percentage of HLA-E positive cells (Figure 2A). In addition, 5GKO.HLA-E.HLA-G cells exhibited significantly higher HLA-E expression than 5GKO.HLA-E cells ($p < 0.05$) (Figure 2C). HLA-G expression was comparable between 5GKO.HLA-G and 5GKO.HLA-E.HLA-G cells (Figures 2B, D). These results indicate that HLA-E molecules are stable and more highly expressed on 5GKO.HLA-E.HLA-G cells than on 5GKO.HLA-E cells. All cells were examined by flow cytometry to ensure HLA-E or HLA-G expression prior to being used in the functional assays.

Enhanced HLA-E expression on 5GKO.HLA-E cells by pulsing exogenous HLA-G VL9 peptides

A recent study indicated that HLA class I signal peptide polymorphism influences surface HLA-E expression as well as NKG2A-HLA-E engagement (41). Surface HLA-E is unstable and is rapidly internalized (42). In 5GKO.HLA-E.HLA-G cells, HLA-E can bind to either HLA-B*07:02 VL9 or HLA-G VL9 peptides. In 5GKO.HLA-E cells, HLA-E can only bind HLA-B*07:02 VL9 peptides. To understand the mechanism by which 5GKO.HLA-E.HLA-G cells exhibited much higher HLA-E expression than 5GKO.HLA-E cells, we determined whether HLA-G VL9 peptide could enhance HLA-E expression on 5GKO.HLA-E cells. 5GKO.HLA-E cells were pulsed with HLA-G VL9 peptides at 25 μM, 50 μM, or 100 μM, and incubated overnight. HLA-E surface expression by flow cytometric analysis revealed that exogenous HLA-G VL9 peptides could significantly increase HLA-E expression on 5GKO.HLA-E cells in a dose dependent manner (Figure 3). This result suggests that HLA-G VL9 peptides can stabilize HLA-E molecules and enhance HLA-E expression on 5GKO.HLA-E cells.

10.3389/fimmu.2023.1217809

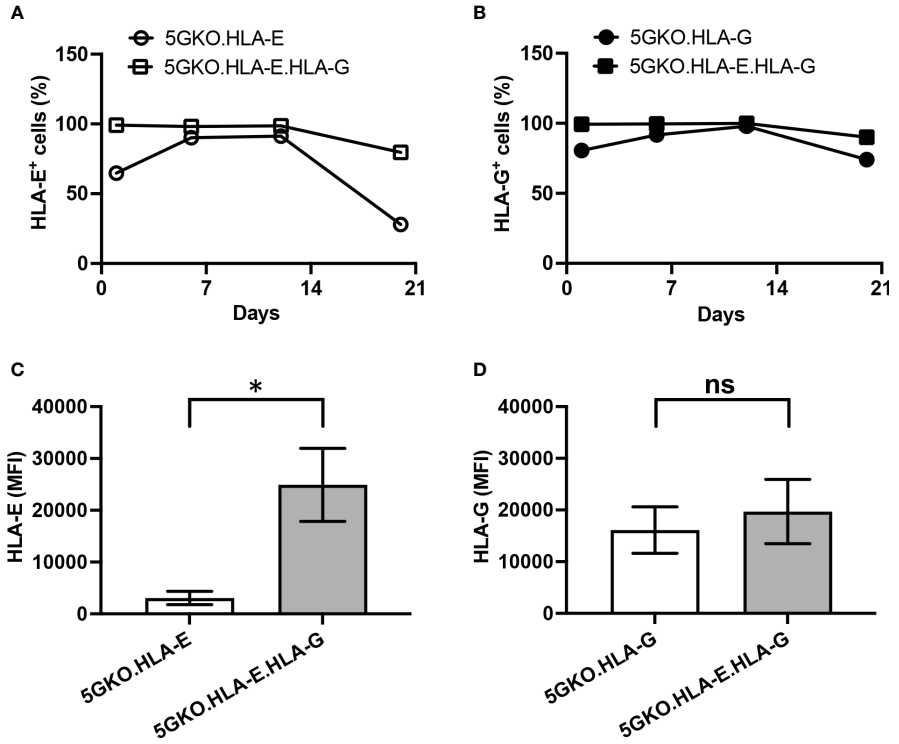


FIGURE 2
Comparison of HLA-E and HLA-G stability and cell surface expression on porcine cells. The percentage of HLA-E positive cells **(A)** and HLA-G positive cells **(B)** in modified pig cells were examined at different time points. **(C)** Abundance of cell surface HLA-E was compared between 5GKO.HLA-E and 5GKO.HLA-E.HLA-G cell lines. **(D)** Abundance of cell surface HLA-G was compared between 5GKO.HLA-G and 5GKO.HLA-E.HLA-G cell lines. Data presented as mean ± SEM. Student's t-test was used to analyze the difference between two groups. ns, not significant; *p < 0.05.

Inhibition of human NK cell degranulation by co-expression of HLA-E and HLA-G on porcine endothelial cells

Human NK cell response to pig cell stimulation was examined by CD107a expression on NK cells. Our previous study demonstrated that 5GKO cells, like WT and TKO (triple-gene knockout, *GGTA1/CMAH/β4galNT2*) cells, could activate human NK cell. Despite the elimination of four xenoantigens (aGal, Neu5Gc, Sda, and SLA-I), 5GKO cells maintained the capability to trigger human NK cell degranulation (36). 5GKO cells were used as a control in this study. The gating strategy to identify the NK cell population ($CD3^-CD56^+$) was shown in Figure 4A. Representative flow plots showing human NK cell degranulation in response to stimulation by each modified cell line as assessed by the

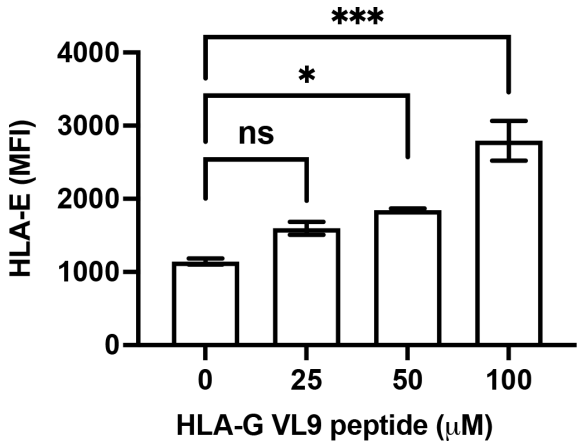


FIGURE 3
Increased HLA-E expression on 5GKO.HLA-E cells after pulsing with HLA-G VL9 peptides. 5GKO.HLA-E cells were incubated with HLA-G VL9 peptides at 25 μM, 50 μM, or 100 μM overnight. HLA-E expression was analyzed by flow cytometry using APC-conjugated mouse anti-HLA-E antibody. ns, not significant; *p < 0.05; ***p<0.001.

10.3389/fimmu.2023.1217809

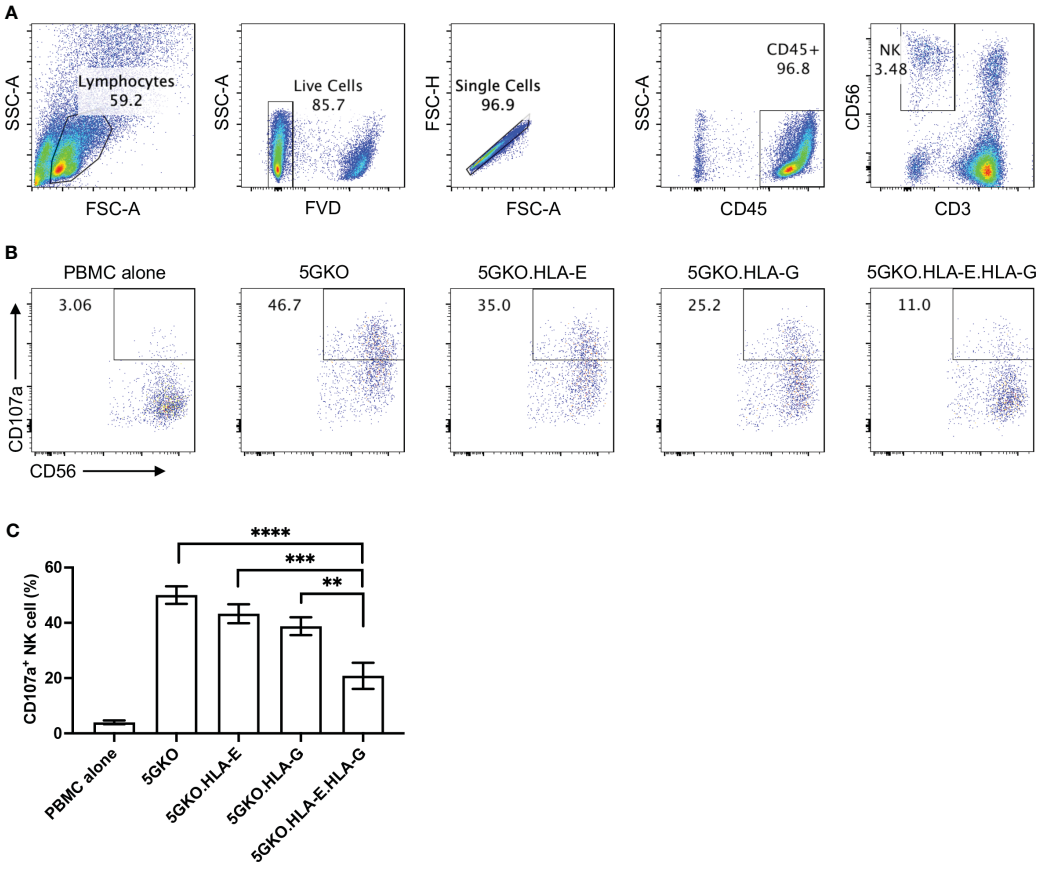


FIGURE 4
Inhibition of human NK cell degranulation by 5GKO cells expressing HLA-E and HLA-G molecules. rhIL-2 treated PBMCs (n=5) were co-cultured with 5GKO, 5GKO.HLA-E, 5GKO.HLA-G, and 5GKO.HLA-E.HLA-G cells for 2 hours. NK cell degranulation was accessed by CD107a surface expression. **(A)** Gating strategy to identify and refine NK cell population. **(B)** Representative flow plots showing NK cell degranulation in response to the simulation of the modified porcine cells by assessing the percentage of CD107a positive cells in $CD3^{-}CD56^{+}$ population. PBMC alone was used as a control. **(C)** Three independent experiments were performed to evaluate NK cell degranulation. Data presented as mean ± SEM. Ordinary one-way ANOVA multiple comparisons test was used to analyze the differences among multiple groups. 5GKO.HLA-E.HLA-G was selected as a control group. **p < 0.01; ***p < 0.001; ****p < 0.0001.

percentage of CD107a positive cells in $CD3^{-}CD56^{+}$ population were shown in Figure 4B. Ordinary one-way ANOVA multiple comparisons indicated that co-expression of HLA-E and HLA-G on 5GKO cells significantly inhibited CD107a expression on human NK cell compared to 5GKO ($p < 0.0001$), 5GKO.HLA-E ($p < 0.001$), and 5GKO.HLA-G ($p < 0.01$) (Figure 4C). Further Student's t-test indicated that HLA-G expression on 5GKO cells significantly inhibited CD107a expression on NK cells compared to 5GKO cells ($p < 0.05$) while HLA-E expression on 5GKO failed to inhibit CD107a expression on NK cells compared to 5GKO cells ($p = 0.1853$).

Human sera antibodies did not react to HLA-E and HLA-G molecules on 5GKO cells

To test whether non-classical HLA class I molecules HLA-E and HLA-G could react to pre-existing HLA class I antibodies, human antibody (IgG and IgM) binding to 5GKO, 5GKO.HLA-E, 5GKO.HLA-G, and 5GKO.HLA-E.HLA-G cells was examined. A total of twenty human sera samples including ten high PRA sera and ten low PRA sera from the patients on the kidney transplant waitlist were used in this experiment. No statistically significant differences in human IgG or IgM binding among groups were observed: IgG with low PRA sera ($p = 0.66$), IgG with high PRA sera ($p = 0.88$), IgM with low PRA sera ($p = 0.88$), and IgM with high PRA sera ($p = 0.75$) (Figures 5A–D). No statistically significant difference was observed among groups in human IgG or IgM binding with the combination of the high PRA and low PRA sera (data not shown). In addition, there were no significant differences between low PRA sera and high PRA sera in IgG binding to 5GKO ($p = 0.65$), 5GKO.HLA-E ($p = 0.67$), 5GKO.HLA-G ($p = 0.56$), 5GKO.HLA-E.HLA-G ($p = 0.53$) (Figure 5E) as well as IgM binding to 5GKO ($p = 0.48$), 5GKO.HLA-E ($p = 0.37$), 5GKO.HLA-G ($p = 0.28$), 5GKO.HLA-E.HLA-G ($p = 0.43$) (Figure 5F). These results indicate that HLA-E and HLA-G on porcine cells do not react to existing antibodies in human sera, even from highly sensitized individuals.

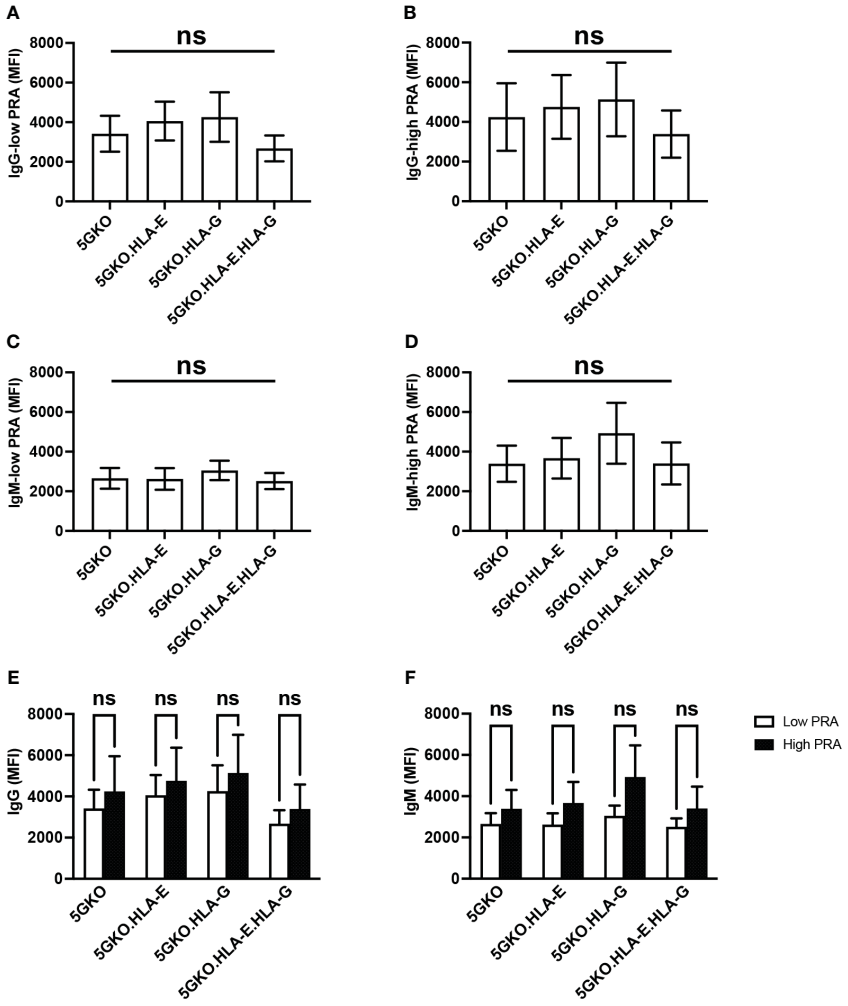


FIGURE 5
Human antibody reactivity to 5GKO cells expressing HLA-E and/or HLA-G molecules. 5GKO, 5GKO.HLA-E, 5GKO.HLA-G, and 5GKO.HLA-E.HLA-G cells were incubated with heat-inactivated human sera (n = 20), and then stained with goat anti-human IgG Alexa Fluor 488 or donkey anti-human IgM Alexa Fluor 647. Each cell line stained with the secondary antibody was used as a negative control. Human antibody binding was assessed by flow cytometry. Data presented as mean ± SEM. Ordinary one-way ANOVA multiple comparisons test was used to analyze the differences in **(A)** IgG binding with low PRA sera, **(B)** IgG binding with high PRA sera, **(C)** IgM binding with low PRA sera, and **(D)** IgM binding with high PRA sera, among multiple groups. **(E)** Comparison of low PRA sera and high PRA sera in IgG binding to individual modified pig cells was analyzed by the Student's t-test. **(F)** Comparison of low PRA sera and high PRA sera in IgM binding to individual modified pig cells was analyzed *via* Student's t-test. ns, not significant.

Discussion

Expressing inhibitory ligands on porcine cells to induce human NK cell tolerance is a practical approach to protect xenografts from human NK cell-mediated destruction (43). We showed that co-expressing HLA-E and HLA-G on a genetically modified 5GKO cell line synergistically reduced human NK cell activation as compared to cells expressing either HLA-E or HLA-G alone as well as 5GKO parental cells. Our study indicated that HLA-E and HLA-G on porcine endothelial cells did not react to human sera antibodies, and the expression of HLA-E and HLA-G is unlikely to elicit antibody-mediated immune responses.

HLA-E and HLA-G are immunoregulatory molecules and their cooperation has been found in immunosuppressive environments, including physiological (immune tolerance at maternal/fetal interface during pregnancy) and pathological (immune evasion of both tumor and viral infection) conditions (44). The role of HLA-E and HLA-G in inhibiting human NK cell activation has been previously demonstrated in xenotransplantation research (10, 16, 17, 26, 33). In the current study, we found that co-expression of HLA-G and HLA-E on porcine endothelial cells effectively inhibited human NK cell degranulation. HLA-E stability and abundance on porcine cells play a key role in inhibiting human NK cell activation. HLA-E expression level was much higher on 5GKO.HLA-E.HLA-G cells, compared to 5GKO.HLA-E cells. HLA-G VL9 peptides were constantly generated from endogenous HLA-G molecules in 5GKO.HLA-E.HLA-G cells, which could stabilize HLA-E molecules and increase HLA-E expression. A surplus of HLA-G

VL9 peptides may be the primary mechanism for the robust increase in cell-surface HLA-E. HLA-E expression on 5GKO.HLA-E cells could also be enhanced by pulsing exogenous HLA-G VL9 peptides in a dose-dependent manner. The stabilized HLA-E-VL9 complexes engage with human NK cell inhibitory receptor NKG2A to protect healthy cells from NK cell-mediated lysis. Previous study indicated that the HLA-E molecules loaded with the HLA-G VL9 peptides exhibited the highest affinity to inhibitory receptor NKG2A compared to the VL9 peptides from HLA-B and HLA-C (32). This magnified inhibition was not associated with HLA-E and HLA-G co-localization on pig cells when co-cultured with human NK cells (unpublished data). In addition, HLA-E and HLA-G interact with different inhibitory receptors on human NK cells through CD94/NKG2-dependent and independent pathways (17, 45). Therefore, co-expression of HLA-E and HLA-G on pig cells leads to a synergistic reduction in human NK cell activation and may provide a novel approach to effectively protect xenografts from human NK cell-mediated cytotoxicity.

In this study, human antibodies (both IgG and IgM) did not react to HLA-E and HLA-G on pig cells. Even across the stratification of human sera with low PRA and high PRA, there was no appreciable increase in antibody binding to HLA-E and HLA-G molecules. These findings suggest that even in highly sensitized individuals, there would likely be no substantial increase in antibody-mediated rejection induced by porcine organs expressing HLA-E and HLA-G.

In human allotransplantation, HLA-E and HLA-G expression can be used to predict transplant outcomes. For example, elevated HLA-G in allografts and in the circulation of recipients was associated with improved graft acceptance in solid organ transplantation (46). In contrast, increased HLA-E expression was found in acute cellular rejection (ACR) biopsies compared to biopsies with no rejection signs, which was correlated with numbers of HLA class I leader peptide mismatches and reduced renal allograft survival (47). Interaction of HLA-E with mismatched HLA class I leader peptides with activating NKG2C receptor may contribute to graft rejection. Recent study demonstrated that mouse and human antibody could bind HLA-E-VL9 complex and enhance NK cell cytotoxicity (48). In xenotransplantation, HLA-E-VL9 complexes could be designed and engineered in pig with the purpose of avoiding antibody binding, facilitating NKG2A interaction, and promoting NK cell inhibition.

The next step will be generating transgenic pigs co-expressing HLA-E and HLA-G. Targeting HLA-E and HLA-G genes to the safe harbor loci in the pig genome can control the copy number of transgene while avoiding undesirable position effects. Studies have shown that the porcine Rosa26 locus and elongation factor 1 alpha (PEF1-alpha) locus are ideal for the integration of transgene for constitutive and ubiquitous expression (49, 50). CRISPR/Cas9-directed targeting of HLA-E and HLA-G constructs to these loci would facilitate transgenic pig production.

In conclusion, our results provide valuable insight into potential mechanisms for overcoming human NK cell-mediated immune rejection in xenotransplantation. Further optimization of this approach, in addition to *in vivo* validation studies, will provide context for the clinical applicability of these GMs in pig-to-human xenotransplantation. The field of xenotransplantation is rapidly progressing, and systematically evaluating potential GMs to optimize pig-to-human compatibility will be crucial to addressing the organ shortage.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, NM_001363567.2 https://www.ncbi.nlm.nih.gov/genbank/, NM_004048.4 https://www.ncbi.nlm.nih.gov/genbank/, NM_005516.6.

Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Review Board (IRB) of Indiana University (IRB#11013).

Author contributions

AC-N, KF, AI, JW, and PL performed experiments, AC-N, WL, WZ, KL, YP, NH, and PL analyzed data. AC-N, WL, ZL, and PL interpreted data. AC-N and PL wrote the paper. DC provided guidance and critical review of the manuscript. PL and BE conceived of the study and provided funding. PL designed experiments and supervised the project. All authors contributed to the article and approved the submitted version.

Funding

This study is supported by NIH NIAID R21AI164002 (PL) and the Ralph W. and Grace M. Showalter Research Trust (PL), an award from the Indiana University School of Medicine. Work on xenotransplantation at Indiana University has been supported by internal funds of the Department of Surgery, in part, with support by the Board of Directors of the Indiana University Health Values Fund for Research Award (VFR-457-Ekser), the Indiana Clinical and Translational Sciences Institute, funded in part by Grant # UL1TR001108 from the National Institutes of Health (NIH),

National Center for Advancing Translational Sciences, Clinical and Translational Sciences Award. This publication was made possible with partial support to an Indiana CTSI MedSTAR Fellow (AC-N) from Grant # UL1TR002529 from the National Institutes of Health, National Center for Advancing Translational Sciences, Clinical and Translational Sciences Award.

Acknowledgments

The authors would like to thank the members of Indiana University Melvin and Bren Simon Cancer Center Flow Cytometry Resource Facility for their outstanding technical support.

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.

Publisher's note

References

1. Ekser B, Li P, Cooper DKC. Xenotransplantation: past, present, and future. *Curr Opin Organ Transplant* (2017) 22(6):513–21. doi: 10.1097/MOT.0000000000000463

2. Cooper DKC, Hara H, Iwase H, Yamamoto T, Li Q, Ezzelarab M, et al. Justification of specific genetic modifications in pigs for clinical organ xenotransplantation. *Xenotransplantation* (2019) 26(4):e12516. doi: 10.1111/xen.12516

3. Cross-Najafi AA, Lopez K, Isidan A, Park Y, Zhang W, Li P, et al. Current barriers to clinical liver xenotransplantation. *Front Immunol* (2022) 13. doi: 10.3389/fimmu.2022.827535

4. Rabin R. In a first, man receives a heart from a genetically altered pig. *New York Times* (2022).

5. Martens GR, Ladowski JM, Estrada J, Wang ZY, Reyes LM, Easlick J, et al. HLA class I-sensitized renal transplant patients have antibody binding to SLA class I epitopes. *Transplantation* (2019) 103(8):1620–9. doi: 10.1097/TP.0000000000002739

6. Ladowski JM, Hara H, Cooper DKC. The role of SLAs in xenotransplantation. *Transplantation* (2021) 105(2):300–7. doi: 10.1097/TP.0000000000003303

7. Navarro-Alvarez N, Yang YG. CD47: a new player in phagocytosis and xenograft rejection. *Cell Mol Immunol* (2011) 8(4):285–8. doi: 10.1038/cmi.2010.83

8. Takeuchi K, Ariyoshi Y, Shimizu A, Okumura Y, Cara-Fuentes G, Garcia GE, et al. Expression of human CD47 in pig glomeruli prevents proteinuria and prolongs graft survival following pig-to-baboon xenotransplantation. *Xenotransplantation* (2021) 28(6):e12708. doi: 10.1111/xen.12708

9. Ide K, Wang H, Tahara H, Liu J, Wang X, Asahara T, et al. Role for CD47-SIRPalpha signaling in xenograft rejection by macrophages. *Proc Natl Acad Sci USA* (2007) 104(12):5062–6. doi: 10.1073/pnas.0609661104

10. Forte P, Baumann BC, Weiss EH, Seebach JD. HLA-e expression on porcine cells: protection from human NK cytotoxicity depends on peptide loading. *Am J Transplant* (2005) 5(9):2085–93. doi: 10.1111/j.1600-6143.2005.00987.x

11. Yazdani S, Callemeyn J, Gazut S, Lerut E, de Loor H, Wevers M, et al. Natural killer cell infiltration is discriminative for antibody-mediated rejection and predicts outcome after kidney transplantation. *Kidney Int* (2019) 95(1):188–98. doi: 10.1016/j.kint.2018.08.027

12. Quan D, Bravery C, Chavez G, Richards A, Cruz G, Copeman L, et al. Identification, detection, and *in vitro* characterization of cynomolgus monkey natural killer cells in delayed xenograft rejection of hDAF transgenic porcine renal xenografts. *Transplant Proc* (2000) 32(5):936–7. doi: 10.1016/S0041-1345(00)01046-0

13. Sivori S, Vacca P, Del Zotto G, Munari E, Mingari MC, Moretta L. Human NK cells: surface receptors, inhibitory checkpoints, and translational applications. *Cell Mol Immunol* (2019) 16(5):430–41. doi: 10.1038/s41423-019-0206-4

14. Szekeres-Bartho J. Regulation of NK cell cytotoxicity during pregnancy. *Reprod BioMed Online* (2008) 16(2):211–7. doi: 10.1016/S1472-6483(10)60576-7

15. Hunt JS, Langat DK, McIntire RH, Morales PJ. The role of HLA-G in human pregnancy. *Reprod Biol Endocrinol* (2006) 4(Suppl 1):S10. doi: 10.1186/1477-7827-4-S1-S10

16. Crew MD. Play it in e or G: utilization of HLA-e and -G in xenotransplantation. *Xenotransplantation* (2007) 14(3):198–207. doi: 10.1111/j.1399-3089.2007.00395.x

17. Sasaki H, Xu XC, Mohanakumar T. HLA-e and HLA-G expression on porcine endothelial cells inhibit xenoreactive human NK cells through CD94/NKG2-dependent and -independent pathways. *J Immunol* (1999) 163(11):6301–5. doi: 10.4049/jimmunol.163.11.6301

18. Robinson J, Barker DJ, Georgiou X, Cooper MA, Flicek P, Marsh SGE. IPD-IMGT/HLA database. *Nucleic Acids Res* (2020) 48(D1):D948–d55. doi: 10.1093/nar/gkz950

19. Edgerly CH, Weimer ET. The past, present, and future of HLA typing in transplantation. *Methods Mol Biol* (2018) 1802:1–10. doi: 10.1007/978-1-4939-8546-3_1

20. Colonna M, Navarro F, Bellon T, Llano M, Garcia P, Samaridis J, et al. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *J Exp Med* (1997) 186(11):1809–18. doi: 10.1084/jem.186.11.1809

21. Colonna M, Samaridis J, Cella M, Angman L, Allen RL, O'Callaghan CA, et al. Human myelomonocytic cells express an inhibitory receptor for classical and nonclassical MHC class I molecules. *J Immunol* (1998) 160(7):3096–100. doi: 10.4049/jimmunol.160.7.3096

22. Goodridge JP, Witt CS, Christiansen FT, Warren HS. KIR2DL4 (CD158d) genotype influences expression and function in NK cells. *J Immunol* (2003) 171(4):1768–74. doi: 10.4049/jimmunol.171.4.1768

23. Kikuchi-Maki A, Yusa S, Catina TL, Campbell KS. KIR2DL4 is an IL-2-regulated NK cell receptor that exhibits limited expression in humans but triggers strong IFN-gamma production. *J Immunol* (2003) 171(7):3415–25. doi: 10.4049/jimmunol.171.7.3415

24. Sheshgiri R, Rouas-Freiss N, Rao V, Butany J, Ramzy D, Krawice-Radanne I, et al. Myocardial HLA-G reliably indicates a low risk of acute cellular rejection in heart transplant recipients. *J Heart Lung Transplant* (2008) 27(5):522–7. doi: 10.1016/j.healun.2008.02.004

25. Brugiere O, Thabut G, Pretolani M, Krawice-Radanne I, Dill C, Herbreteau A, et al. Immunohistochemical study of HLA-G expression in lung transplant recipients. *Am J Transplant* (2009) 9(6):1427–38. doi: 10.1111/j.1600-6143.2009.02650.x

26. Forte P, Pazmany L, Matter-Reissmann UB, Stussi G, Schneider MK, Seebach JD. HLA-G inhibits rolling adhesion of activated human NK cells on porcine endothelial cells. *J Immunol* (2001) 167(10):6002–8. doi: 10.4049/jimmunol.167.10.6002

27. Forte P, Matter-Reissmann UB, Strasser M, Schneider MK, Seebach JD. Porcine aortic endothelial cells transfected with HLA-G are partially protected from xenogeneic human NK cytotoxicity. *Hum Immunol* (2000) 61(11):1066–73. doi: 10.1016/S0198-8859(00)00202-0

28. Weiss EH, Lilienfeld BG, Müller S, Müller E, Herbach N, Kessler B, et al. HLA-e/human beta2-microglobulin transgenic pigs: protection against xenogeneic human anti-pig natural killer cell cytotoxicity. *Transplantation* (2009) 87(1):35–43. doi: 10.1097/TP.0b013e318191c784

29. Lilienfeld BG, Crew MD, Forte P, Baumann BC, Seebach JD. Transgenic expression of HLA-e single chain trimer protects porcine endothelial cells against human natural killer cell-mediated cytotoxicity. *Xenotransplantation* (2007) 14(2):126–34. doi: 10.1111/j.1399-3089.2007.00378.x

30. Crew MD, Cannon MJ, Phanavanh B, Garcia-Borges CN. An HLA-e single chain trimer inhibits human NK cell reactivity towards porcine cells. *Mol Immunol* (2005) 42(10):1205–14. doi: 10.1016/j.molimm.2004.11.013

31. Lee N, Goodlett DR, Ishitani A, Marquardt H, Geraghty DE. HLA-e surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J Immunol* (1998) 160(10):4951–60. doi: 10.4049/jimmunol.160.10.4951

32. Vales-Gomez M, Reyburn HT, Erskine RA, Lopez-Botet M, Strominger JL. Kinetics and peptide dependency of the binding of the inhibitory NK receptor CD94/NKG2-a and the activating receptor CD94/NKG2-c to HLA-e. *EMBO J* (1999) 18 (15):4250–60. doi: 10.1093/emboj/18.15.4250

33. Matsunami K, Miyagawa S, Nakai R, Yamada M, Shirakura R. Modulation of the leader peptide sequence of the HLA-e gene up-regulates its expression and down-regulates natural killer cell-mediated swine endothelial cell lysis. *Transplantation* (2002) 73(10):1582–9. doi: 10.1097/00007890-200205270-00010

34. Laird CT, Burdorf L, French BM, Kubicki N, Cheng X, Braileanu G, et al. Transgenic expression of human leukocyte antigen-e attenuates GalKO.hCD46 porcine lung xenograft injury. *Xenotransplantation* (2017) 24(2). doi: 10.1111/xen.12294

35. Puga Yung G, Bongoni AK, Pradier A, Madelon N, Papaserafeim M, Sfriso R, et al. Release of pig leukocytes and reduced human NK cell recruitment during ex vivo perfusion of HLA-e/human CD46 double-transgenic pig limbs with human blood. *Xenotransplantation* (2018) 25(1). doi: 10.1111/xen.12357

36. Li P, Walsh JR, Lopez K, Isidan A, Zhang W, Chen AM, et al. Genetic engineering of porcine endothelial cell lines for evaluation of human-to-pig xenoreactive immune responses. *Sci Rep* (2021) 11(1):13131. doi: 10.1038/s41598-021-92543-y

37. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. *J Immunol Methods* (2004) 294(1-2):15–22. doi: 10.1016/j.jim.2004.08.008

38. Kim J, Phan MT, Kweon S, Yu H, Park J, Kim KH, et al. A flow cytometry-based whole blood natural killer cell cytotoxicity assay using overnight cytokine activation. *Front Immunol* (2020) 11:1851. doi: 10.3389/fimmu.2020.01851

39. Liu B, Yang GX, Sun Y, Tomiyama T, Zhang W, Leung PSC, et al. Decreased CD57 expression of natural killer cells enhanced cytotoxicity in patients with primary sclerosing cholangitis. *Front Immunol* (2022) 13:912961. doi: 10.3389/fimmu.2022.912961

40. Hofle J, Trenkner T, Kleist N, Schwane V, Vollmers S, Barcelona B, et al. Engagement of TRAIL triggers degranulation and IFNgamma production in human natural killer cells. *EMBO Rep* (2022) 23(8):e54133. doi: 10.15252/embr.202154133

41. Lin Z, Bashirova AA, Viard M, Garner L, Quastel M, Beiersdorfer M, et al. HLA class I signal peptide polymorphism determines the level of CD94/NKG2-HLA-E-mediated regulation of effector cell responses. *Nat Immunol* (2023) 24(7):1087–97. doi: 10.1038/s41590-023-01523-z

42. He W, Gea-Mallorqui E, Colin-York H, Fritzsche M, Gillespie GM, Brackenridge S, et al. Intracellular trafficking of HLA-e and its regulation. *J Exp Med* (2023) 220(8):e20221941. doi: 10.1084/jem.20221941

43. Lopez KJ, Cross-Najafi AA, Farag K, Obando B, Thadasina D, Isidan A, et al. Strategies to induce natural killer cell tolerance in xenotransplantation. *Front Immunol* (2022) 13:941880. doi: 10.3389/fimmu.2022.941880

44. Morandi F, Pistoia V. Interactions between HLA-G and HLA-e in physiological and pathological conditions. *Front Immunol* (2014) 5:394. doi: 10.3389/fimmu.2014.00394

45. Navarro F, Llano M, Bellon T, Colonna M, Geraghty DE, Lopez-Botet M. The ILT2(LIR1) and CD94/NKG2A NK cell receptors respectively recognize HLA-G1 and HLA-e molecules co-expressed on target cells. *Eur J Immunol* (1999) 29(1):277–83. doi: 10.1002/(SICI)1521-4141(199901)29:01<277::AID-IMMU277>3.0.CO;2-4

46. Liu S, Bos NA, Verschuuren EAM, van Baarle D, Westra J. Biological characteristics of HLA-G and its role in solid organ transplantation. *Front Immunol* (2022) 13:902093. doi: 10.3389/fimmu.2022.902093

47. Guberina H, Rebmann V, Wagner B, da Silva Nardi F, Dziallas P, Dolff S, et al. Association of high HLA-e expression during acute cellular rejection and numbers of HLA class I leader peptide mismatches with reduced renal allograft survival. *Immunobiology* (2017) 222(3):536–43. doi: 10.1016/j.imbio.2016.10.021

48. Li D, Brackenridge S, Walters LC, Swanson O, Harlos K, Rozbesky D, et al. Mouse and human antibodies bind HLA-e-leader peptide complexes and enhance NK cell cytotoxicity. *Commun Biol* (2022) 5(1):271. doi: 10.1038/s42003-022-03183-5

49. Li X, Yang Y, Bu L, Guo X, Tang C, Song J, et al. Rosa26-targeted swine models for stable gene over-expression and cre-mediated lineage tracing. *Cell Res* (2014) 24 (4):501–4. doi: 10.1038/cr.2014.15

50. Sun WS, Yang H, No JG, Lee H, Lee N, Lee M, et al. Select porcine elongation factor 1alpha sequences mediate stable high-level and upregulated expression of heterologous genes in porcine cells in response to primate serum. *Genes (Basel)* (2021) 12(7):1046. doi: 10.3390/genes12071046

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 04 December 2023
DOI 10.3389/fimmu.2023.1293762

OPEN ACCESS

EDITED BY
Lisha Mou,
Shenzhen Second People's Hospital, China

REVIEWED BY
Simona Marzorati,
NanoPhoria S.r.l., Italy
Pradeep Shrestha,
University of Texas MD Anderson Cancer Center, United States
Shannon Townsend,
Washington University in St. Louis, United States
Valia Bravo-Egana,
Augusta University, United States

*CORRESPONDENCE
Jun-bo Liang
drliangjb@163.com
Shao-wei Li
li_shaowei81@hotmail.com

RECEIVED 13 September 2023
ACCEPTED 20 November 2023
PUBLISHED 04 December 2023

CITATION
Chen Q-d, Liu L, Zhao X-h, Liang J-b and Li S-w (2023) Challenges and opportunities in the islet transplantation microenvironment: a comprehensive summary of inflammatory cytokine, immune cells, and vascular endothelial cells.
Front. Immunol. 14:1293762.
doi: 10.3389/fimmu.2023.1293762

Challenges and opportunities in the islet transplantation microenvironment: a comprehensive summary of inflammatory cytokine, immune cells, and vascular endothelial cells

Qi-dong Chen[1], Long Liu[2], Xiao-hong Zhao[3], Jun-bo Liang[1]* and Shao-wei Li[4,5]*

[1]Taizhou Hospital, Zhejiang University School of Medicine, Taizhou, Zhejiang, China, [2]Department of Hepatobiliary and Pancreatic Surgery, Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China, [3]Department of Pharmacy, Taizhou Hospital, Zhejiang University , Taizhou, Zhejiang, China, [4]Department of Gastroenterology, Taizhou Hospital of Zhejiang Province affiliated to Wenzhou Medical University, Linhai, Zhejiang, China, [5]Key Laboratory of Minimally Invasive Techniques & Rapid Rehabilitation of Digestive System Tumor of Zhejiang Province, Taizhou Hospital Affiliated to Wenzhou Medical University, Linhai, Zhejiang, China

It is now understood that islet transplantation serves as a β-cell replacement therapy for type 1 diabetes. Many factors impact the survival of transplanted islets, especially those related to the microenvironment. This review explored microenvironmental components, including vascular endothelial cells, inflammatory cytokines, and immune cells, and their profound effects on post-islet transplantation survival rates. Furthermore, it revealed therapeutic strategies aimed at targeting these elements. Current evidence suggests that vascular endothelial cells are pivotal in facilitating vascularization and nutrient supply and establishing a new microcirculation network for transplanted islets. Consequently, preserving the functionality of vascular endothelial cells emerges as a crucial strategy to enhance the survival of islet transplantation. Release of cytokines will lead to activation of immune cells and production and release of further cytokines. While immune cells hold undeniable significance in regulating immune responses, their activation can result in rejection reactions. Thus, establishing immunological tolerance within the recipient's body is essential for sustaining graft functionality. Indeed, future research endeavors should be directed toward developing precise strategies for modulating the microenvironment to achieve higher survival rates and more sustained transplantation outcomes. While acknowledging certain limitations inherent to this review, it provides valuable insights that can guide further exploration in the field of islet transplantation. In conclusion, the microenvironment plays a paramount role in islet transplantation. Importantly, we discuss novel

perspectives that could lead to broader clinical applications and improved patient outcomes in islet transplantation.

KEYWORDS

islets transplantation, microenvironment, inflammatory cytokines, vascular endothelial cells, immune cells

1 Introduction

Type I diabetes mellitus (T1DM) results from the absence of islet β cells, typically due to autoimmune attacks or surgical pancreas removal (1). The typical symptoms of high blood sugar due to insulin deficiency often manifest rapidly and include increased urination, thirst, weight loss, abdominal discomfort, and headaches. Without appropriate replacement therapy, patients may ultimately develop microvascular complications, ketoacidosis, and even death (2, 3). In 1921, Franklin Banting's discovery of insulin revolutionized the management of T1DM, turning it into a manageable chronic condition. The development of rapid and long-acting insulins and the clinical use of insulin pumps combined with continuous glucose monitoring (CGM) have led to remarkable therapeutic advancements (4). However, the reliance on CGM, insulin pumps, dietary control, and increased physical activity places significant financial and psychological stress on patients and their families. Moreover, these exogenous therapies, CGMs and insulin pumps have a delayed detection and control of blood glucose levels, whereas pancreatic cells are able to detect blood glucose levels more quickly and accurately and deliver precisely measured amounts of insulin (5). Moreover, although intensified insulin treatment regimens can ameliorate glycated hemoglobin levels, they do not provide protection against diabetes complications (6). Pancreas transplantation becomes a consideration when patients face severe metabolic complications, incapacitating problems with exogenous insulin therapy or failure of insulin-based management to prevent acute complications (7). By transplanting an entire vascularized pancreas, we can restore the natural balance between blood glucose and insulin (8). Nevertheless, this approach remains challenging, primarily due to immunological considerations. While the matching of the donor pancreas to the recipient's HLA type is a desirable goal to prevent hyperacute and acute rejection (9). The paramount consideration lies in ensuring compatibility, meaning the absence of pre-existing HLA antibodies specific to the donor's HLA antigens in the recipient (10). Furthermore, the presence of postoperative complications is often a contributing factor to transplant failure (7). Hence, the concept of islet transplantation emerged as a less invasive and complication-prone cellular therapy (11). Unfortunately, many issues must be addressed to improve survival after islet transplantation, including islet viability, effective implantation, islet function, and immune response resulting in islet damage (12). Currently, islet transplantation primarily involves intrahepatic transplantation into the portal vein due to its accessibility and lower morbidity (13). Regrettably, immediate islet loss post-transplantation can be as high as 50%-70% (14). It is widely thought that transplanted islets are directly exposed to blood in the liver and its complex microenvironment, significantly contributing to this early loss. Factors include immediate blood-mediated inflammatory responses, immune reactions, and the impact of angiogenesis on the transplanted islets (15, 16).

This paper aims to summarize the influence of the microenvironment on islet survival post-transplantation, with a particular focus on inflammatory cytokines, vascular endothelial cells, immune cells, and potential strategies to address these challenges.

2 The process of islet transplantation

Currently, the primary source of pancreatic islets for clinical transplantation is deceased donors. An ideal donor should meet the following criteria: age between 20 and 50 years, BMI less than 30 kg/m^2, and HbA1c less than 6.5% (17–20).

Once the pancreas is excised, it should be promptly preserved in a cold storage solution to ensure the quality of preservation. To obtain clinically usable islet preparations, pancreatic tissue must undergo enzymatic digestion using a mixture of collagenase and protease enzymes. This process disperses acinar cells while minimizing damage to the islets.

Following the completion of enzymatic digestion, pancreatic islets must undergo purification, as impure islet preparations exhibit reduced functionality compared to their purified counterparts. Moreover, infusing larger tissue volumes from insufficiently purified islets can lead to increased portal vein pressure, raising the risk of portal vein thrombosis. Post-purification, the isolated islets can be cultured in a suitable medium for 24-72 hours to assess their functionality and viability (21).

The final pancreatic islet cell product is suspended in a transplantation culture medium and loaded into sterile infusion bags containing 70 units of heparin per kilogram of recipient body weight (20). During the surgical process, access to the portal vein system is achieved through percutaneous or minimally invasive abdominal approaches, allowing for direct infusion of the islets into the portal vein system. The islets are then retained within the small

portal vein branches within the liver parenchyma, ultimately establishing microvascular blood supply (22).

3 Microenvironmental factors in islet transplantation

3.1 Vascular endothelial cells

3.1.1 Relationship between vascular endothelial cell and islet survival

Endothelial cells (ECs) are a predominant cell type within the pancreatic islets, organized into a precisely regulated and morphologically distinct microcirculation network that facilitates a high degree of vascularization within the pancreatic tissue. As shown in Figure 1, in human islets of Langerhans (with a diameter of 40-60 μm), β-cells are located at the core, while blood vessels are situated in the periphery. In larger islets, micro vessels penetrate and branch within the islet's interior, and the insulin produced by these β-cells is transported to the peripheral circulation through the microvasculature within the islets (23). The survival and functionality of endothelial cells are therefore paramount for rapid and efficient blood perfusion after pancreatic islet transplantation (24, 25). However, when the islets were cultured prior to transplantation, the ECs within the islets decreased rapidly and disappeared after 7 days of culture (26).

In the days following pancreatic islet transplantation, processes of angiogenesis, neovascularization, and vascular reconstruction swiftly ensue. These dynamic events primarily involve the participation of donor endothelial cells, recipient's local vascular cells, and recruited cells from the bone marrow (27, 28). Ultimately, the vascular system formed within the transplanted islets represents a mosaic of cells from both donor and recipient origins. The reconstitution of blood flow within the transplanted islets occurs within 7-14 days, yet the post-reconstruction vascular density exhibits a reduction compared to native islets, amounting to 24% of the native pancreatic islet vascular density (29, 30).

Furthermore, an increasing body of research suggests that endothelial cells play a constructive role within the pancreatic islet microenvironment, engaging in crosstalk with β-cells (31, 32). Endothelial cells function as endocrine cells, releasing various active molecules through distinct molecular pathways, such as Hepatocyte Growth Factor (HGF), Thrombospondin-1 (TSP-1), laminin, collagen, among others, inducing nearby β-cells to differentiate, proliferate, survive, and enhance insulin secretion (33–35). Consequently, promoting post-islet transplantation

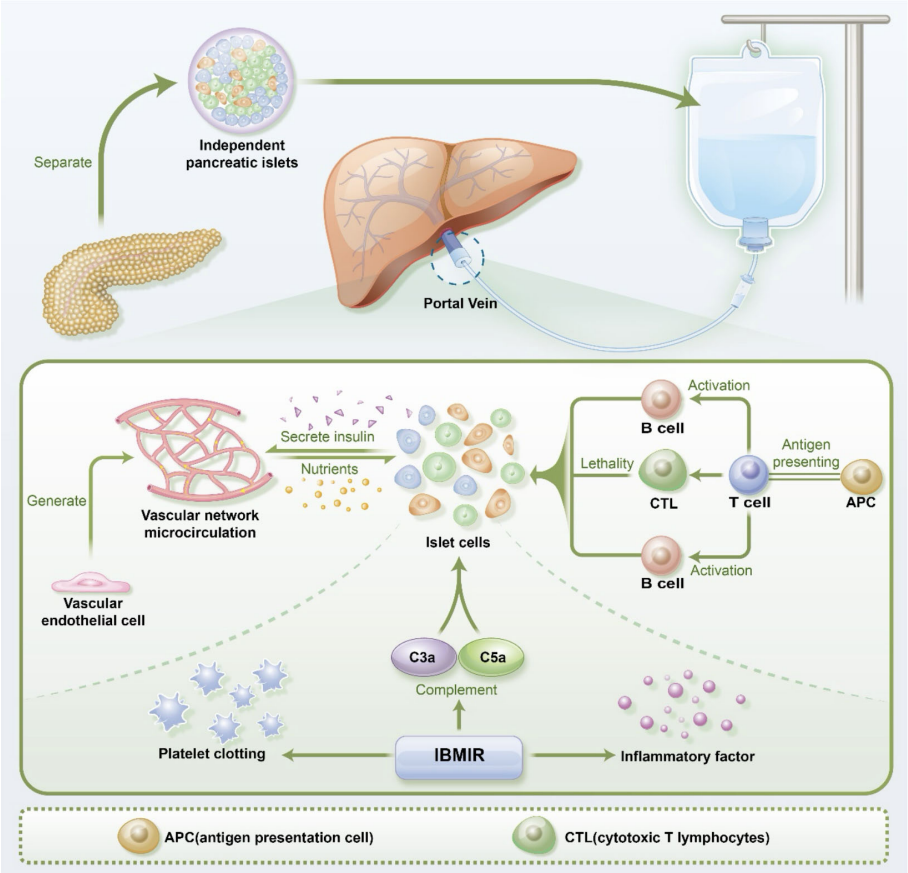


FIGURE 1
The process of pancreatic islet transplantation and the microenvironmental challenges faced by pancreatic islets, including vascular endothelial cells, immune cells, and IBMIR(immediate blood-mediated inflammatory response). APC(antigen presentation cell) CTL(cytotoxic T lymphocytes).

vascularization may emerge as a novel therapeutic target for diabetes treatment.

Vascular endothelial growth factor (VEGF), generated by β cells within islets, plays a pivotal role in regulating islet vascular development and vascular homeostasis. In ECs, VEGF induces cell migration and proliferation and maintains fenestrations. Insufficient VEGF levels have been correlated with decreased capillary density and vascular permeability within islets, subsequently impairing their functionality (36). However, studies indicate that endogenous angiogenic factors produced by transplanted islets might be inadequate to induce angiogenesis in the early post-transplantation period (37). For instance, Montazeri et al. demonstrated that a porous collagen scaffold loaded with VEGF within rat pancreatic islet transplants facilitated vascular generation and improved graft functionality (38). Similarly, Yin et al., utilizing VEGF-conjugated alginate material to encapsulate transplanted islets, exhibited sustained angiogenic promotion upon subcutaneous transplantation (39). However, an excess of vascular endothelial growth factor (VEGF) is not universally beneficial, as its overexpression can lead to vascular dysfunction and pancreatic islet impairment (40). Therefore, investigating the appropriate VEGF concentration within the microenvironment is of paramount importance for facilitating early vascular formation in transplanted islets.

In addition to leveraging the angiogenic properties of VEGF to stimulate vascular development, cell-based adjunct therapy during transplantation represents a promising strategy for enhancing the process of pancreatic islet revascularization. Given the limited survival characteristics and plasticity of mature endothelial cells, endothelial progenitor cells (EPCs) emerge as an optimal choice. EPCs, originating from the bone marrow, possess the capacity to migrate to sites of tissue injury or ischemia and actively participate in angiogenesis and endothelial regeneration (41). Moreover, the utilization of autologous blood for EPC isolation can effectively mitigate the risk of rejection. Research conducted by Daniella et al. has demonstrated that the co-transplantation of EPCs significantly augments the engraftment rate of transplanted islets and improves initial glycemic control (42). Studies by Liza et al. further underscore that islet grafts encapsulating endothelial progenitor cells exhibit markedly enhanced blood perfusion and oxygen tension compared to control grafts (43). Beyond endothelial cells, specific cell types have also been identified with angiogenesis-promoting capabilities. Research suggests that M2-type macrophages can also stimulate neovascularization in transplanted islets, reduce cellular apoptosis, and enhance islet graft survival (44).

These findings suggest that vascular endothelial cells can improve the survival and function of transplanted islets by promoting angiogenesis.

3.1.2 The effect of angiogenesis on pancreatic oxygen supply

Despite being in direct contact with the bloodstream within the portal vein, early vascularization deficiencies in transplanted islets lead to their reliance on surface oxygen diffusion rather than direct arterial perfusion with oxygenated blood (45). Research by K. E. Dionne et al. demonstrated that isolated Langerhans islets exhibited diminished insulin secretion due to hypoxia. This reduction in insulin secretion correlated with the presence of intra- and extra-islet Oxygen partial pressure gradients, resulting in a radial decline in islet cell exposure to low Oxygen partial pressure levels from the periphery to the core (46). Furthermore, under hypoxic conditions, aerobic glucose metabolism shifts to anaerobic glycolysis, ultimately triggering caspase-3 activation and islet cell apoptosis (47). Therefore, complete vascularization of transplanted islets is crucial for providing an adequate oxygen supply to reverse these phenomena.

In a study by Haofei Li et al., GelMA/HepMA/VEGF scaffolds were found to recruit human umbilical vein endothelial cells, fostering a rich vascular network around the scaffold. This augmented neovascular network significantly increased subcutaneous oxygen content, enhancing islet vitality, especially in the early stages of islet transplantation (48). Liza Grapensparr et al. enveloped human islet transplants with endothelial progenitor cells derived from umbilical cord blood and placed them in the subcapsular space of the kidney in non-obese diabetic/severe combined immunodeficiency mice. Four weeks post-transplantation, blood flow perfusion and oxygen tension of the grafts were assessed using laser Doppler flowmetry and Clark microelectrodes, respectively. Notably, islet transplants with incorporated endothelial progenitor cells exhibited significantly higher blood flow perfusion and oxygen tension compared to control grafts (43).

In conclusion, recruiting vascular endothelial cells and promoting angiogenesis are believed to ameliorate the oxygen supply situation for transplanted islets.

3.1.3 The role of microcirculation on pancreatic islets

The microcirculation within pancreatic islet primarily consists of vascular endothelial cells, that facilitates the delivery of nutrients and waste clearance in response to glucose fluctuations. It achieves this while avoiding significant changes in hydrostatic pressure to preserve the integrity of islet capillary exchange (49). Traditionally, islets were considered independent of the surrounding exocrine tissue, lacking an integrated capillary network connecting the endocrine and exocrine pancreas. Blood flow within islets was believed to be unidirectional, with the sole connection between the endocrine and exocrine systems being the islet-acinar portal vein through which blood exits the islet and enters the exocrine tissue (50). Currently, there are three main models regarding the concept of islet perfusion: 1. Non-β cells being perfused before β-cells, allowing other endocrine cells to influence downstream β-cell function. 2. β-cells being perfused before other endocrine cells. In this scenario, β-cells are given a relatively high perfusion priority, so they dominate the function of the islets. 3. No distinct perfusion order. However, recent studies have indicated that both mouse and human islets are not confined to the closed "glomerulus-like" structure but rather exhibit an open arrangement where islet capillaries continuously merge with capillaries of the exocrine

pancreas (51, 52). Blood flow within the islet microcirculation holds significance for islet development and the regulation of the islet hormone network, with microcirculatory abnormalities impeding insulin production and accelerating the progression of diabetes (53). In a study by Chieko Ihoriya et al. changes in islet microcirculation were investigated by administering varying doses of Angiotensin II or Angiotensin I receptor blockers via intravenous injection. Their study revealed that islet microcirculatory blood flow decreased after islet vasoconstriction, subsequently leading to reduced glucose-stimulated insulin secretion (54).

Hence, during the isolation of islets from the pancreas for transplantation purposes, the detachment not only physically separates islets from their complete capillary network but also introduces potential differences in function and structure compared to islets within the native environment. These differences may impact the functionality of transplanted islets.

In conclusion, the reconstruction of islet microcirculation is of great significance to improve the survival and reproduce the function of transplanted islets.

3.2 Inflammatory cytokine

3.2.1 Inflammatory cytokines and islet damage

The early loss of transplanted islet vitality due to early inflammatory responses poses a significant challenge to the long-term survival rate of pancreatic islet transplantation, akin to other organ or tissue transplants. In fact, it is estimated that up to 80% of transplanted islets are lost during the initial inflammatory reaction (55).

As shown in Figure 1, the immediate blood-mediated inflammatory response (IBMIR) plays a key role in this process. Traditional pancreatic islet transplantation via the portal vein exposes islets directly to the blood, triggering IBMIR. IBMIR is initiated by strong activation of the coagulation cascade, where negatively charged surface of the islets activate the intrinsic coagulation pathway (56), and islet-expressed tissue factor (TF) induces the extrinsic coagulation pathway (57). This cascade activates thrombin, prompting endothelial cells to release pro-inflammatory cytokines such as interleukin-6 (IL-6) and IL-8, leading to the recruitment and accumulation of nearby neutrophils and macrophages. Simultaneously, macrophages release an array of pro-inflammatory factors, including interferon-gamma (IFN-γ), IL-1β, IL-6, and IL-8, sustaining the inflammatory response (58). The islets themselves also secrete numerous inflammatory factors like monocyte chemoattractant protein-1 (MCP-1), IFN-γ, IFN-γ-inducible protein-10 (IP-10), IL-6, and IL-8 due to hypoxia and stress (58). These pro-inflammatory factors further trigger inflammatory reactions, escalating islet cell apoptosis and causing damage to transplanted islets.

Additionally, the activation of the complement system is a crucial aspect of IBMIR, reflected in increased complement concentrations in the serum of pancreatic islet transplant recipients (59). The activation of complement proteins C3a and C5a leads to leukocyte recruitment and accumulation, upregulation of endothelial and platelet adhesion molecules, and the generation of reactive oxygen species (ROS) (60). ROS can activate the NF-κB signaling pathway through protein, lipid, and nucleic acid degradation, ultimately inducing β-cell death (61).

In summary, recipients of pancreatic islet transplants generate inflammatory responses that, influenced by various inflammatory cytokines, lead to early loss and functional deactivation of transplanted islets.

3.2.2 The potential of anti-inflammatory therapy to improve islet graft survival

Considering that early inflammatory responses within the transplantation microenvironment significantly contribute to the early loss and functional decline of transplanted islets, it becomes imperative to enhance anti-inflammatory management during the peri-transplant period becomes imperative. Based on a 20-year cohort from a Canadian single-center study, the combined use of IL-1 receptor antagonist (anakinra) and TNF inhibitor (etanercept) during transplantation has shown potential to increase the likelihood of sustained graft survival (62).

Numerous preclinical studies also support the perspective of anti-inflammatory treatment to improve graft survival. Quercetin, as an inflammation-modulating compound, holds promise in ameliorating post-transplant islet injury. In an *in vitro* study, quercetin-3-o-glucoside (C3G) treatment significantly reduced inflammatory markers IL-1β and NLRP3 protein expression in grafts (63). Bilirubin is the ultimate product of heme metabolism, and numerous clinical studies have demonstrated an inverse correlation between plasma bilirubin levels and various diseases (64–67). In animal models, bilirubin has exhibited anti-inflammatory activity, including in conditions such as endotoxemia, sepsis, and ischemia-reperfusion injury (68–71). Its mechanisms of action include the inhibition of inflammatory cell infiltration and the reduction of nitric oxide (NO) production (72–74). Therefore, due to its anti-inflammatory and cellular protective properties, bilirubin is considered a potential drug for protecting transplanted islets and mitigating inflammatory damage. In research by Zhu et al., exposure of INS-1 cells, simulating rat insulinoma, to cytokine-induced inflammation (IL-1β, TNF-α, and IFN-γ) resulted in cellular damage. Bilirubin, at appropriate lower concentrations, effectively mitigated INS-1 cell viability reduction and reduced cytokine-induced cell apoptosis, thereby protecting insulin secretion functionality (75). Additionally, pre-conditioning with purified bilirubin at the isolation stage improved overall islet survival by downregulating the expression of pro-inflammatory genes (MCP-1, TNF-α) (76). Antonio Citro et al. validated in a mouse experiment that CXCR1/2 inhibitors reduced leukocyte recruitment induced by transplantation, significantly prolonging graft rejection onset in a syngeneic allograft environment (77).

Moreover, the damage response of donor islets during separation and purification activates graft inflammation, exerting negative impacts. Tissue factor (TF) is considered a “danger signal,” highly present on the islet surface, and can elicit IBMIR by activating the extrinsic coagulation pathway. Clinical outcomes of

islet transplantation have been directly correlated with TF expression levels, suggesting that TF blockade represents a novel therapeutic avenue to enhance the survival rate of type 1 diabetes islet transplantation (78). Strategies to inhibit TF function have been explored, including monoclonal antibodies, inactivated FVIIa factor, small-molecule inhibitors, and siRNA (78). Another "danger signal," high mobility group box-1 protein (HMGB1), released from donor-derived islets, often signifies adverse outcomes in transplanted islets (79). Research by Nobuhide Matsuoka et al. indicated that treatment with HMGB1-specific antibodies prevented early islet graft loss and suppressed the production of IFN-γ by NKT cells and Gr-1(+)CD11b(+) cells (80). Eun Hee Jo et al. employed the HMGB1 receptor antagonist, HMGB1 A box, as an innovative approach for the encapsulation of isolated pancreatic islets, which were subsequently co-cultured with macrophages. The findings demonstrated a notable decrease in TNF-α secretion by macrophages co-cultured with encapsulated islets compared to non-encapsulated ones. Moreover, following transplantation of the encapsulated islets into diabetic mice, there was a twofold increase in islet survival rates (81). Thus, targeting the pathways mediated by HMGB1 offers potential intervention for early islet loss.

Activation of the complement system is integral to IBMIR since it serves as a crucial mediator for the release of inflammatory cytokines. Complement-derived anaphylatoxins C3a and C5a released upon IBMIR activation are believed to participate in leukocyte recruitment and infiltration. Therefore, drugs targeting complement activation also hold potential therapeutic effects to inhibit inflammation and improve transplantation outcomes. Complement C5a receptor inhibitor peptide (C5aIP) weakens the link between complement and coagulation cascades by inhibiting the upregulation of white blood cell tissue factor expression, specifically in the liver (82). Importantly, the soluble complement receptor 1 inhibitor sCR1 and TP10 exert protective effects on post-transplant islets (83, 84).

However, drugs targeting only a fraction of IBMIR processes are unlikely to block all elements of the reaction (i.e., coagulation, complement activation, production of pro-inflammatory mediators); thus, a combination of multiple drugs is necessary to enhance post-transplant islet survival.

3.3 Immune cells

3.3.1 Effector immune cells and regulatory immune cells

Effector immune cells refer to a specific class of cells within the immune system that play a crucial role when the body faces infection or immune challenges. These cells are primarily responsible for eliminating infectious agents or abnormal cells to maintain an effective immune response. Key effector immune cells include cytotoxic T cells (CD8+ T cells), macrophages, natural killer (NK) cells, plasma cells, and CD4+ T helper cells.

Cytotoxic CD8+ T cells play a pivotal role in graft rejection reactions. CD8+ T cells directly eliminate cells presenting non-self-antigens by releasing cytotoxic molecules, such as granules and perforins, or by inducing apoptosis through cell surface interactions, like the binding of FAS ligand (also known as CD95L) on T cells to FAS receptors on target cells (85). Activated CD8+ T cells that infiltrate transplanted organs also induce the activation of macrophages, particularly through the expression of proinflammatory cytokines, such as IFN-γ (86).

Natural killer cells (NK cells) are innate immune lymphocytes that control the spread and subsequent tissue damage caused by various types of tumors and microbial infections through MHC-independent cytotoxicity (87). Recent research indicates that NK cells also act as regulatory cells interacting with dendritic cells, macrophages, T cells, and endothelial cells, modulating immune responses accordingly (88).

Macrophages are typically characterized as proinflammatory and exhibit M1 polarization during acute rejection reactions, producing proinflammatory cytokines, which result in direct cell damage and coordination of the proinflammatory immune response (89). Their major role is phagocytosis, recognizing damaged allogeneic transplant tissue through pattern recognition receptors, such as Toll-like receptors. As antigen-presenting cells, macrophages can present alloantigens in MHC class II molecules, thereby promoting adaptive immune responses (90).

Plasma cells are another type of effector immune cell derived from B cells and form the cornerstone of humoral immunity. They enable the body to combat foreign invaders, not only by neutralizing pathogens but also by performing various effector functions, including the regulation of hypersensitivity reactions, activation of the complement cascade, and modulation of mucosal microbial communities. However, their activity can be problematic in solid organ transplantation (91). In transplantation, plasma cells can produce donor-specific antibodies (DSAs), which, by activating the complement system, lead to acute and chronic rejection, resulting in vascular damage and graft loss (92). The impact of DSAs has been extensively assessed in various solid organ transplantations (93–95).

Regulatory immune cells constitute a specialized class of cells within the immune system, primarily tasked with maintaining immune homeostasis and preventing excessive immune responses. These cells play a pivotal role in regulating immune responses, suppressing autoimmunity, and limiting inflammatory processes. They include regulatory T cells, regulatory B cells, suppressive macrophages, and NK cells.

Treg cells, a subset of CD4+ T cells, are a crucial component of regulatory immune cells. Treg cells can be categorized as thymus derived Treg cells, which develop in the thymus. Their differentiation, maintenance, and functionality are tightly regulated by the expression of the transcription factor Foxp3 (Forkhead box P3). Another pathway for Treg cell generation occurs in peripheral blood cells under the influence of antigen stimulation and the appropriate combination of cytokines, including IL-2 and transforming growth factor (TGF)-beta (96).

The interest in regulatory B cells (Bregs) dates to the 1970s, with evidence suggesting that B cells can modulate the immune system by producing "suppressive" antibodies. Regulatory B cells (Bregs) discovered in mice and humans have been shown to downregulate inflammation associated with various pathological processes,

including autoimmune diseases, transplant rejection, anti-tumor responses, and infections. These cells have the capacity to produce anti-inflammatory cytokines such as IL-10, TGF-beta, and IL-35, and are considered to have the foundational capacity to induce regulatory T cells (Tregs), contributing to their regulatory potential (97).

Macrophages can exhibit both protective and pathological functions. In transplantation, macrophage activation initially occurs due to tissue damage associated with ischemia-reperfusion and may lead to early graft injury. In contrast, alternatively activated macrophages can suppress the production of proinflammatory cytokines by classically activated macrophages and facilitate wound healing and tissue repair. This repair process is highly critical in the early post-transplantation period, as wound healing helps reestablish tissue homeostasis (98).

CD4+ T helper cells play a crucial role in immune rejection. They coordinate the activation of other immune cells, such as B cells and cytotoxic T cells, to enhance the immune response against allogeneic substances. These CD4+ T cells possess the ability to produce and release various cytokines, including interferon-gamma (IFN-γ) and interleukin-2 (IL-2). Additionally, CD4+ T cells actively interact with B cells, promoting the generation of antibodies and thereby strengthening humoral immunity (99, 100).

3.3.2 Relationship between immune cells and damage to transplanted islets

Like most organ transplants, immune rejection is a common occurrence in pancreatic islet transplant recipients, contributing to the loss of islet graft function (101).

Immunological reactions manifest as unexplained hyperglycemia, unexpected reduction in C-peptide levels, susceptibility events, and heightened immunological risk. It is widely acknowledged that the human immune system comprises both the innate and adaptive immune systems, featuring immune cells like macrophages, dendritic cells (DCs), natural killer (NK) cells, B cells, and T cells. Macrophages engage primarily in phagocytosis, while DCs can be categorized into lymphoid tissue-resident and non-lymphoid tissue-resident subsets, with their principal role being antigen presentation. They express major histocompatibility complex (MHC) class I and II antigens, thereby activating CD8+ cytotoxic T lymphocytes (CTL) and CD4+ helper cells. NK cells, part of the innate immune system, are known for their ability to eliminate virus-infected or cancer cells, and they can also contribute to adaptive immune responses by releasing pro-inflammatory cytokines such as IFN-γ. The cytotoxicity of NK cells is finely regulated by activating and inhibitory receptors, including human killer cell Ig-like receptors (KIRs) and mouse c-type lectin-like family receptors (102). B lymphocytes are chiefly responsible for antibody production. When the islets are transplanted into the recipient, B lymphocytes can recognize the antigens that are foreign to the organ and produce antibodies to attack these antigens, causing damage to the transplanted tissue. T lymphocytes exist in various subtypes, such as helper T cells (Th1), Th2, Th17, and regulatory T cells (Tregs). Immune rejection following transplantation initiates with the infiltration of innate immune cells, especially macrophages, into the transplanted islets, followed by donor-specific lymphocyte responses involving CD4+ and CD8+ T cells and B cells.

The activation of T cells primarily occurs through three pathways: First, DCs can directly migrate from the transplanted islets to secondary lymphoid organs, where they present donor MHC molecules, thereby activating allogeneic T cell responses. In the semi-direct pathway, DCs and other antigen-presenting cells (APCs) can phagocytose allogeneic cells, present allogeneic MHC molecules on their surface, and subsequently activate T cells. Allogeneic proteins are degraded by recipient APCs, and allogeneic peptides are presented on self-MHC molecules. These allogeneic peptide-self-MHC complexes can be recognized by T cell receptors (103).

The exogenous peptides or antigens are initially internalized and processed by antigen-presenting cells, such as dendritic cells and macrophages. These antigen-presenting cells bind antigenic fragments with major histocompatibility complex (MHC) molecules, forming MHC-antigen complexes. T cells recognize these MHC-antigen complexes through their T cell receptors (TCRs) (104). CD8+ T cells bind MHC-I-antigen complexes, while CD4+ T cells bind MHC II-antigen complexes, through their respective TCRs, subsequently activating T cells and leading to T cell proliferation and differentiation. Through the interaction of CD40-CD40L, activated T cells engage in vital crosstalk with B cells, initiating a cascade of signaling events. This interaction propels the further development of B cells, transforming them into cells with the capacity to generate antibodies, thereby strengthening the humoral immune response (105). This process holds significance in the context of organ transplantation, enhancing immune responses against allogeneic substances and potentially correlating with transplant immune rejection. In the context of transplant rejection, T cells can distinguish MHC and foreign antigens within the transplanted organ, triggering a rejection response aimed at disrupting the integrity of the transplanted organ (106, 107). Upon activation, CD8+ T cells secrete cytotoxic molecules, including perforin and granzyme B, leading to the direct killing of transplanted islet cells as presented in Figure 1. In contrast, CD4+ T cells do not directly harm grafts; instead, they enhance the function of CD8+ cells and secrete a range of inflammatory factors, such as TNF-α and IFN-γ, resulting in local inflammatory cell infiltration and damage to β cells in the transplanted islets (108). Furthermore, the interaction between CD4 + T cells and B cells promotes the activation of B cells, leading to their differentiation into antibody-producing cells known as Plasma B cells. These Plasma B cells produce antibodies, ultimately resulting in damage to the transplanted pancreatic islets (106).

3.3.3 Potential applications for suppression of immune rejection

In conventional approaches, clinicians often employ immunosuppressive drugs (ISDs) to inhibit the proliferation and function of effector T cells, thereby attenuating the body's rejection response (109). Early immunosuppressive regimens primarily consisted of corticosteroids, azathioprine, and cyclosporine (110).

However, this therapeutic approach yielded insulin independence in only approximately 10% of patients within a year. In recent years, the development of the "Edmonton protocol" has significantly improved clinical outcomes of pancreatic islet transplantation. This novel immunosuppressive regimen involves sirolimus, low-dose tacrolimus, and induction with anti-interleukin-2 receptor antibodies. Remarkably, this regimen achieves a high rate of insulin independence, with approximately 80% of patients becoming insulin-independent within a year (111). Unfortunately, this protocol necessitates lifelong medication, which diminishes patients' quality of life, and raises the risk of various adverse reactions, such as susceptibility to infections and potential secondary malignancies (112). In addition, because ISDs are absorbed through the intestine and islets are infiltrated directly into the bloodstream via the portal vein, ISDs would have a direct toxic effect on pancreatic islet beta cells, further reducing the survival of transplanted islets (113, 114). Consequently, the ultimate goal of pancreatic islet transplantation is to attain donor-specific immune tolerance. Indeed, there is an urgent need for new strategies to avoid lifelong use of immunosuppressive agents, enhance graft survival rates, and improve secretion function.

T cell depletion represents a promising strategy. Recent studies have shown that anti-CD3 induction therapy, by depleting a significant number of T cells, holds great potential for promoting immune suppression. An anti-CD3 immunotoxin based on diphtheria toxin has been demonstrated to induce tolerance (115). María M Coronel et al. devised an immunosuppressive regimen involving programmed death ligand-1 mediated by biomaterials to treat an allogeneic islet transplantation model. This approach was characterized by the enrichment of CD206+ programmed death 1+ macrophages and the depletion of cytotoxic T cells in the graft microenvironment (116). In addition, the induction of stable mixed chimerism by bone marrow transplantation is widely recognized as a reliable and robust method of tolerance induction (117). By mimicking central tolerance, it is possible to achieve almost complete elimination of donor-specific T cells in recipients. Selective long-term depletion of donor-specific T-cell clones in the host and donor-specific graft tolerance have been achieved in preclinical rodent models (118). However, considerations of toxicity associated with recipient preconditioning and the threat of graft-versus-host disease have hampered the clinical application of this method.

Regulatory T cells (Tregs), capable of suppressing the activation and function of effector T cells, play a crucial role in maintaining immune homeostasis (119). In recent years, the characteristics of Treg cells have been harnessed to inhibit immune rejection post-transplantation. In this regard, Dario Gerace et al. engineered stem cell-derived islet cells to secrete interleukin-10 (IL-10), transforming growth factor-β (TGF-β), and modified IL-2 in addition to targeting human leukocyte antigen (HLA) and PD-L1, recruiting Tregs to enhance immune tolerance within the graft microenvironment. Results demonstrated that engineered human islet cell grafts transplanted into non-obese diabetic (NOD) mice resisted allogeneic rejection for up to 8 weeks (120). Besides, Evelina et al. co-transplanted islets with a plasmid encoding the chemokine CCL22 into the muscle of MHC-mismatched mice, resulting in localized accumulation of Tregs due to the expression and secretion of pCCL22 in muscle cells. Consequently, the population of effector T lymphocytes around the islets decreased significantly, and the onset of immune rejection was markedly delayed compared to the control group (121). In conclusion, Ying Li et al. designed a poly (lactic-co-glycolic acid) microparticle (PLGA MP) system for the local release of TGF-β1, which, when co-incubated with CD4+ T cells *in vitro*, efficiently generated antigen-specific induced Tregs (iTregs) with potent immunosuppressive functions, providing substantial protection for the graft (122).

While Treg cell therapy continues to evolve, it indiscriminately suppresses the immune system without achieving a permanent resolution of certain diseases. Transgenic Tregs offer significant promise in addressing these issues. CAR-Treg cells, an emerging immunotherapy, employ CAR (Chimeric Antigen Receptor) technology, a synthetic receptor that empowers immune cells to selectively recognize and target specific antigens. This allows regulatory T cells to modulate immune responses and reduce inflammation to prevent the immune response from damaging the graft (123). Boardman et al. discovered that, in a human skin xenograft transplant model using immunodeficient mice, adoptively transferred CAR-Tregs were more effective in alleviating allogeneic immune-mediated skin damage caused by peripheral blood mononuclear cell transplants compared to polyclonal Tregs. *In vitro* experiments demonstrated that CAR-Tregs produced anti-inflammatory interleukin-10 (IL-10) in the presence of alloantigen (124). These findings highlight the potential benefits of CAR-Tregs in graft-specific immunosuppression. The therapeutic potential of antigen specific Tregs has been confirmed in numerous autoimmune diseases, including T1D, colitis, transplant rejection, and hemophilia (125–128).

Graft modification prior to transplantation is an excellent strategy to reduce rejection and improve clinical applicability. Ali Zafar et al. maintained isolated porcine pancreatic islet cells in a three-dimensional rotating cell culture system and allowed them to aggregate with human amniotic epithelial cells. In a porcine-mouse islet transplantation model, the stem cell-modified islets had better insulin secretion than natural islets, and the allogeneic response to them by CD4+ T cells was significantly reduced (129). This provides a new way of thinking about xenogeneic islet transplantation.

In addition to immune response inhibition, some researchers have employed islet encapsulation methods to physically isolate cells from the host using a barrier that restricts the infiltration of immune cells and antibodies while allowing the penetration of oxygen, nutrients, and insulin. Interestingly, Yesl Jun et al. prepared collagen-alginate composite fiber-encapsulated islets using a microfluidic platform to simulate the natural islet microenvironment. The results demonstrated that composite fiber-encapsulated islets exhibited higher viability and more stable insulin secretion compared to free islets (130). Su et al. designed a hydrogel network and presented inhibitory peptides against the IL-1 receptor on the surface of pancreatic islet cells and showed that

these peptide-modified hydrogels were effective in protecting the encapsulated cells from specific T-lymphocyte attack (131). These results suggest that encapsulating cells and tissues in hydrogels with anti-inflammatory or immunosuppressive agents may be a novel strategy to improve the function of cells and tissues in transplantation and tissue engineering.

Due to individual variations, specific treatment regimens may not be universally applicable. In the management of transplant patients, the use of biomarkers contributes to achieving genuinely personalized therapy. Immunological biomarkers offer a better reflection of the activity of drugs (or drug combinations), going beyond mere concentration measurements and providing greater value compared to pharmacokinetic assessments for immunosuppressive agents (132). Brunet et al. conducted a comprehensive review of the application of biomarkers in transplantation, discussing three categories of biomarkers: [1] those related to rejection risk (allograft reactivity/tolerance), [2] those reflecting individual responses to immunosuppressive agents, and [3] those associated with graft dysfunction (133). The objective of individualized immunosuppression is to minimize the toxicity associated with immunosuppressive regimens, with the potential to enhance long-term allograft survival without compromising short-term allograft survival (134). Thus, optimizing immunosuppression holds significant importance in improving the clinical prognosis of pancreatic islet transplant recipients. However, current research on biomarkers remains in its preliminary stages, with numerous limitations. The immune system exhibits significant variability among different individuals, posing a challenge in the quest for universal biomarkers applicable to all patients. The immune status is a dynamic and multifaceted process influenced by various factors. Variability in biomarkers over time and in different environments may hinder accurate predictions of immune states in certain circumstances. Furthermore, the mechanisms underlying transplant immune rejection are intricate, involving multiple cell types and signaling pathways. Thus, relying on a single or limited set of biomarkers may inadequately capture the comprehensive assessment of immune status (135–137).

3.3.4 Immune checkpoint blockade

Antibody-mediated immune checkpoint blockade represents a revolutionary cancer immunotherapy. These same mechanisms can be reutilized to control destructive allogeneic immune responses in the transplant setting. Currently, one of the most effective and durable immunotherapies in clinical use revolves around the programmed cell death-1 (PD-1) pathway. The PD-1/PD-L1 axis plays a pivotal role in regulating alloimmune responses in the transplant environment (138). Experimental models of fully mismatched allogeneic heart transplants have demonstrated the necessity of intact PD-1/PD-L1 interactions and blocking PD-1 results in prolonged rejection times (139). Overexpression of the immune checkpoint protein programmed death-ligand 1 (PD-L1) protects human islet-like organ allografts, enabling them to maintain glucose homeostasis for 50 days in immune-competent diabetic mice (140). Shirwan and colleagues have engineered a synthetic biomaterial platform for local delivery of a chimeric streptavidin-affibody/programmed cell death-1 ligand 1 (SA-PD-L1) protein to reprogram local immune responses to transplanted islets. In a mouse model of diabetes, only when mice received SA-PD-L1-presenting biomaterial and brief rapamycin treatment could local induction of allograft acceptance be achieved. Immunological profiling showed an increase in regulatory T cells and anergic cells following SA-PD-L1 hydrogel delivery (138).

The CD47/SIRPα pathway is involved in regulating innate and adaptive immune responses. This system negatively regulates macrophage activation and phagocytosis, adhesion, platelet activation, and antibody-dependent cell-mediated cytotoxicity and phagocytosis (141–143). It has been reported that the interaction between CD47 expressed on dendritic cells (DCs) and antibodies or SIRPα expressed on T cells can inhibit DC activation and their secretion of pro-inflammatory cytokines, leading to a weakened T cell response (144, 145). Shirwan and colleagues have constructed a chimeric structure, SA-CD47, containing the extracellular domain of CD47 modified to include a streptavidin (SA) moiety. In a murine marginal mass islet transplant model, SA-CD47-engineered islets demonstrated superior engraftment and function compared to the SA control group (146).

CTLA-4, cytotoxic T-lymphocyte-associated antigen 4, is a critical immune checkpoint protein and a negative regulator. CTLA-4 exerts its inhibitory effects by interacting with B7 molecules on antigen-presenting cells, thereby suppressing T cell activation (147). Zhang and colleagues employed inkjet-based bioprinting technology to precisely deliver trace amounts of murine CTLA4/Fc fusion protein into human decellularized dermal matrix scaffolds. These scaffolds were co-transplanted with allogeneic islets under the renal capsule, establishing an immune-regulatory microenvironment around the allogeneic islets, achieving long-term engraftment of low-dose allogeneic islet cells (148).

Fas (CD95) and Fas ligand (FasL) play significant roles in immune function, including inducing cell apoptosis and regulating T cell activation (149). Fas deficiency in mice results in abnormal accumulation of antigen-specific T cells during chronic viral infections and under steady-state conditions (150, 151). Furthermore, loss-of-function mutations in genes encoding Fas and FasL lead to autoimmune lymphoproliferative syndrome (ALPS), suggesting the role of Fas and FasL in controlling lymphocyte proliferation and maintaining immune tolerance (152). Michael Skoumal and colleagues modified allogeneic islets with biotin and transiently displayed SA-FasL on their surface in a peritoneal fat pad using a micro-porous scaffold. After a short course (15 days) of rapamycin treatment, they observed sustained survival (153).

4 Non- hepatic transplant site

The portal vein/liver is currently the preferred site for clinical islet transplantation, accounting for 90% of clinical islet transplantations. However, early extensive islet damage due to the influence of the portal vein microenvironment has been observed following

transplantation. The development of alternative transplantation sites may make it possible to implement strategies to modulate the islet microenvironment in ways not currently feasible in the liver, thereby improving survival and transplantation outcomes (154, 155).

Benjamin and colleagues suggest further research into the subcapsular space below the kidney as a site for clinical islet transplantation. This anatomical location may avoid early IBMIR-mediated damage to the islets and may promote vascular reconstruction (156).

Intramuscular and subcutaneous spaces are important candidates, as the transplantation and biopsy procedures are simple, minimally invasive, and have fewer complications. Although these sites are characterized by low vascularity and hypoxia, many experimental trials have been conducted to enhance outcomes of intramuscular and subcutaneous islet transplantation, with a focus on early vascularization of the transplanted islets (157).

Lonnie D. Shea and colleagues report the use of a proteolytically degradable synthetic hydrogel functionalized with vasculogenic factors for localized delivery, engineered to deliver islet grafts to extrahepatic transplant sites through *in situ* gelation under physiological conditions. These hydrogels induced differences in vascularization and innate immune responses among subcutaneous, small bowel mesentery, and epididymal fat pad transplant sites, with improved vascularization and reduced inflammation observed at the epididymal fat pad site. This biomaterial-based strategy improved the survival, engraftment, and function of individual pancreatic islet grafts (158).

The spleen has been studied as a candidate site for islet transplantation for a long time. Its advantages include physiological insulin drainage and immune regulation, which have recently been demonstrated to contribute to islet regeneration. Additionally, the spleen serves as a reservoir for mesenchymal stem cells that aid in tissue repair (159).

Zhen Liang and colleagues successfully implanted human pluripotent stem cell-derived islets into the abdominal transplantation site - the rectus sheath of eight non-human primates (5 males and 3 females), improving blood glucose control in diabetic primates (160).

These results suggest that non-hepatic sites as transplantation targets are worthy of further exploration.

5 Conclusion

In this comprehensive review, we have meticulously summarized the impact of the microenvironment on pancreatic islet transplant survival. We emphasize the pivotal role of inflammatory cytokines, vascular endothelial cells, and immune cells in enhancing overall transplant outcomes.

Preserving the functionality of vascular endothelial cells is the cornerstone for improving transplant survival. Controlling the levels of inflammatory factors helps to reduce the damage of the graft caused by the early inflammatory response; however, further research is needed to explore how to maintain defense against pathogenic microorganisms while suppressing undesired immune response against the graft to ensure the safe survival of the transplants. In the realm of immune cells, achieving a delicate balance is of paramount importance. Efforts are being made toward advances in individualized immunosuppression, immune modulation therapies, cell engineering, novel drug formulations, and immune checkpoint blockade for more precise immune regulation and suppression. Additionally, non-hepatic transplant sites also warrant further exploration.

In conclusion, the microenvironment profoundly influences the success of pancreatic islet transplantation. Future research should prioritize the fine-tuning of the microenvironment to enhance transplant efficacy.

Author contributions

Q-DC: Writing – original draft. LL: Writing – original draft. X-HZ: Writing – original draft. J-BL: Writing – review & editing. S-WL: Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported in part by Medical Science and Technology Project of Zhejiang Province (2024KY1788), Program of Taizhou Science and Technology Grant (23ywa33), Major Research Program of Taizhou Enze Medical Center Grant (19EZZDA2), Open Project Program of Key Laboratory of Minimally Invasive Techniques and Rapid Rehabilitation of Digestive System Tumor of Zhejiang Province (21SZDSYS01).

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.

Publisher's note

References

1. Buschard K. The etiology and pathogenesis of type 1 diabetes - A personal, non-systematic review of possible causes, and interventions. *Front Endocrinol* (2022) 13:876470. doi: 10.3389/fendo.2022.876470

2. Syed FZ. Type 1 diabetes mellitus. *Ann Internal Med* (2022) 175:Itc33–itc48. doi: 10.7326/AITC202203150

3. Katsarou A, Gudbjörnsdottir S, Rawshani A, Dabelea D, Bonifacio E, Anderson BJ, et al. Type 1 diabetes mellitus. *Nat Rev Dis Primers* (2017) 3:17016. doi: 10.1038/nrdp.2017.16

4. Maiorino MI, Signoriello S, Maio A, Chiodini P, Bellastella G, Scappaticcio L, et al. Effects of continuous glucose monitoring on metrics of glycemic control in diabetes: A systematic review with meta-analysis of randomized controlled trials. *Diabetes Care* (2020) 43:1146–56. doi: 10.2337/dc19-1459

5. Daneman D. Type 1 diabetes. *Lancet (London England)* (2006) 367:847–58. doi: 10.1016/S0140-6736(06)68341-4

6. White SA, Shaw JA, Sutherland DE. Pancreas transplantation. *Lancet (London England)* (2009) 373:1808–17. doi: 10.1016/S0140-6736(09)60609-7

7. Dean PG, Kukla A, Stegall MD, Kudva YC. Pancreas transplantation. *BMJ (Clinical Res ed.)* (2017) 357(2017):j1321. doi: 10.1136/bmj.j1321

8. Dholakia S, Oskrochi Y, Easton G, Papalois V. Advances in pancreas transplantation. *J R Soc Med* (2016) 109:141–6. doi: 10.1177/0141076816636369

9. Brayman KL, Sutherland DE. Factors leading to improved outcome following pancreas transplantation–the influence of immunosuppression and HLA matching. *Transplant Proc* (1992) 24:91–5.

10. Opelz G, Lenhard V. Immunological factors influencing renal graft survival. *Annu Rev Med* (1983) 34:133–44. doi: 10.1146/annurev.me.34.020183.001025

11. Marfil-Garza BA, Hefler J, Verhoeff K, Lam A, Dajani K, Anderson B, et al. Pancreas and islet transplantation: comparative outcome analysis of a single-centre cohort over 20-years. *Ann Surg* (2023) 277:672–80. doi: 10.1097/SLA.0000000000005783

12. Rother KI, Harlan DM. Challenges facing islet transplantation for the treatment of type 1 diabetes mellitus. *J Clin Invest* (2004) 114:877–83. doi: 10.1172/JCI200423235

13. Wojtusciszyn A, Branchereau J, Esposito L, Badet L, Buron F, Chetboun M, et al. Indications for islet or pancreatic transplantation: Statement of the TREPID working group on behalf of the Société francophone du diabète (SFD), Société francaise d'endocrinologie (SFE), Société francophone de transplantation (SFT) and Société française de néphrologie - dialyse - transplantation (SFNDT). *Diabetes Metab* (2019) 45:224–37. doi: 10.1016/j.diabet.2018.07.006

14. Toso C, Zaidi H, Morel P, Armanet M, Andres A, Pernin N, et al. Positron-emission tomography imaging of early events after transplantation of islets of Langerhans. *Transplantation* (2005) 79:353–5. doi: 10.1097/01.TP.0000149501.50870.9D

15. Delaune V, Berney T, Lacotte S, Toso C. Intraportal islet transplantation: the impact of the liver microenvironment. *Transplant international: Off J Eur Soc Organ Transplant* (2017) 30:227–38. doi: 10.1111/tri.12919

16. Nalbach L, Roma LP, Schmitt BM, Becker V, Körbel C, Wrublewsky S, et al. Improvement of islet transplantation by the fusion of islet cells with functional blood vessels. *EMBO Mol Med* (2021) 13:e12616. doi: 10.15252/emmm.202012616

17. Kaddis JS, Danobeitia JS, Niland JC, Stiller T, Fernandez LA. Multicenter analysis of novel and established variables associated with successful human islet isolation outcomes. *Am J transplantation: Off J Am Soc Transplant Am Soc Transplant Surgeons* (2010) 10:646–56. doi: 10.1111/j.1600-6143.2009.02962.x

18. Hanley SC, Paraskevas S, Rosenberg L. Donor and isolation variables predicting human islet isolation success. *Transplantation* (2008) 85:950–5. doi: 10.1097/TP.0b013e3181683df5

19. Nano R, Clissi B, Melzi R, Calori G, Maffi P, Antonioli B, et al. Islet isolation for allotransplantation: variables associated with successful islet yield and graft function. *Diabetologia* (2005) 48:906–12. doi: 10.1007/s00125-005-1725-3

20. Shapiro AM, Pokrywczynska M, Ricordi C. Clinical pancreatic islet transplantation. *Nat Rev Endocrinol* (2017) 13:268–77. doi: 10.1038/nrendo.2016.178

21. Szot GL, Lee MR, Tavakol MM, Lang J, Dekovic F, Kerlan RK, et al. Successful clinical islet isolation using a GMP-manufactured collagenase and neutral protease. *Transplantation* (2009) 88:753–6. doi: 10.1097/TP.0b013e3181b443ae

22. Ahearn AJ, Parekh JR, Posselt AM. Islet transplantation for Type 1 diabetes: where are we now? *Expert Rev Clin Immunol* (2015) 11:59–68. doi: 10.1586/1744666X.2015.978291

23. Bosco D, Armanet M, Morel P, Niclauss N, Sgroi A, Muller YD, et al. Unique arrangement of alpha- and beta-cells in human islets of Langerhans. *Diabetes* (2010) 59:1202–10. doi: 10.2337/db09-1177

24. Bellacen K, Kalay N, Ozeri E, Shahaf G, Lewis EC. Revascularization of pancreatic islet allografts is enhanced by α-1-antitrypsin under anti-inflammatory conditions. *Cell Transplant* (2013) 22:2119–33. doi: 10.3727/096368912X657701

25. Loganathan G, Graham ML, Radosevich DM, Soltani SM, Tiwari M, Anazawa T, et al. Factors affecting transplant outcomes in diabetic nude mice receiving human, porcine, and nonhuman primate islets: analysis of 335 transplantations. *Transplantation* (2013) 95:1439–47. doi: 10.1097/TP.0b013e318293b7b8

26. Nyqvist D, Köhler M, Wahlstedt H, Berggren PO. Donor islet endothelial cells participate in formation of functional vessels within pancreatic islet grafts. *Diabetes* (2005) 54:2287–93. doi: 10.2337/diabetes.54.8.2287

27. Contreras JL, Smyth CA, Eckstein C, Bilbao G, Thompson JA, Young CJ, et al. Peripheral mobilization of recipient bone marrow-derived endothelial progenitor cells enhances pancreatic islet revascularization and engraftment after intraportal transplantation. *Surgery* (2003) 134:390–8. doi: 10.1067/msy.2003.250

28. Brissova M, Fowler M, Wiebe P, Shostak A, Shiota M, Radhika A, et al. Intraislet endothelial cells contribute to revascularization of transplanted pancreatic islets. *Diabetes* (2004) 53:1318–25. doi: 10.2337/diabetes.53.5.1318

29. Henriksnäs J, Lau J, Zang G, Berggren PO, Köhler M, Carlsson PO. Markedly decreased blood perfusion of pancreatic islets transplanted intraportally into the liver: disruption of islet integrity necessary for islet revascularization. *Diabetes* (2012) 61:665–73. doi: 10.2337/db10-0895

30. Jansson L, Carlsson PO. Graft vascular function after transplantation of pancreatic islets. *Diabetologia* (2002) 45:749–63. doi: 10.1007/s00125-002-0827-4

31. Narayanan S, Loganathan G, Dhanasekaran M, Tucker W, Patel A, Subhashree V, et al. Intra-islet endothelial cell and β-cell crosstalk: Implication for islet cell transplantation. *World J Transplant* (2017) 7:117–28. doi: 10.5500/wjt.v7.i2.117

32. Cao Z, Wang X. The endocrine role between β cells and intra-islet endothelial cells. *Endocrine J* (2014) 61:647–54. doi: 10.1507/endocrj.EJ14-0045

33. Olsson R, Carlsson PO. The pancreatic islet endothelial cell: emerging roles in islet function and disease. *Int J Biochem Cell Biol* (2006) 38:710–4. doi: 10.1016/j.biocel.2006.02.004

34. Olerud J, Mokhtari D, Johansson M, Christoffersson G, Lawler J, Welsh N, et al. Thrombospondin-1: an islet endothelial cell signal of importance for β-cell function. *Diabetes* (2011) 60:1946–54. doi: 10.2337/db10-0277

35. Guney MA, Petersen CP, Boustani A, Duncan MR, Gunasekaran U, Menon R, et al. Connective tissue growth factor acts within both endothelial cells and beta cells to promote proliferation of developing beta cells. *Proc Natl Acad Sci U.S.A.* (2011) 108:15242–7. doi: 10.1073/pnas.1100072108

36. Brissova M, Shostak A, Shiota M, Wiebe PO, Poffenberger G, Kantz J, et al. Pancreatic islet production of vascular endothelial growth factor–a is essential for islet vascularization, revascularization, and function. *Diabetes* (2006) 55:2974–85. doi: 10.2337/db06-0690

37. Carlsson PO, Palm F, Andersson A, Liss P. Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site. *Diabetes* (2001) 50:489–95. doi: 10.2337/diabetes.50.3.489

38. Montazeri L, Hojjati-Emami S, Bonakdar S, Tahamtani Y, Hajizadeh-Saffar E, Noori-Keshtkar M, et al. Improvement of islet engrafts by enhanced angiogenesis and microparticle-mediated oxygenation. *Biomaterials* (2016) 89:157–65. doi: 10.1016/j.biomaterials.2016.02.043

39. Yin N, Han Y, Xu H, Gao Y, Yi T, Yao J, et al. VEGF-conjugated alginate hydrogel prompt angiogenesis and improve pancreatic islet engraftment and function in type 1 diabetes. *Materials Sci engineering. C Materials Biol Appl* (2016) 59:958–64. doi: 10.1016/j.msec.2015.11.009

40. Cai Q, Brissova M, Reinert RB, Pan FC, Brahmachary P, Jeansson M, et al. Enhanced expression of VEGF-A in β cells increases endothelial cell number but impairs islet morphogenesis and β cell proliferation. *Dev Biol* (2012) 367:40–54. doi: 10.1016/j.ydbio.2012.04.022

41. Roberts N, Jahangiri M, Xu Q. Progenitor cells in vascular disease. *J Cell Mol Med* (2005) 9:583–91. doi: 10.1111/j.1582-4934.2005.tb00490.x

42. Penko D, Rojas-Canales D, Mohanasundaram D, Peiris HS, Sun WY, Drogemuller CJ, et al. Endothelial progenitor cells enhance islet engraftment, influence β-cell function, and modulate islet connexin 36 expression. *Cell Transplant* (2015) 24:37–48. doi: 10.3727/096368913X673423

43. Grapensparr L, Christoffersson G, Carlsson PO. Bioengineering with endothelial progenitor cells improves the vascular engraftment of transplanted human islets. *Cell Transplant* (2018) 27:948–56. doi: 10.1177/0963689718759474

44. Li Y, Ding X, Tian X, Zheng J, Ding C, Li X, et al. Islet transplantation modulates macrophage to induce immune tolerance and angiogenesis of islet tissue in type I diabetes mice model. *Aging* (2020) 12:24023–32. doi: 10.18632/aging.104085

45. Suszynski TM, Avgoustiniatos ES, Papas KK. Intraportal islet oxygenation. *J Diabetes Sci Technol* (2014) 8:575–80. doi: 10.1177/1932296814525827

46. Dionne KE, Colton CK, Yarmush ML. Effect of hypoxia on insulin secretion by isolated rat and canine islets of Langerhans. *Diabetes* (1993) 42:12–21. doi: 10.2337/diab.42.1.12

47. Emamaullee JA, Shapiro AM. Interventional strategies to prevent beta-cell apoptosis in islet transplantation. *Diabetes* (2006) 55:1907–14. doi: 10.2337/db05-1254

48. Li H, Shang Y, Feng Q, Liu Y, Chen J, Dong H. A novel bioartificial pancreas fabricated via islets microencapsulation in anti-adhesive core-shell microgels and

macroencapsulation in a hydrogel scaffold prevascularized in vivo. *Bioact Mater* (2023) 27:362–76. doi: 10.1016/j.bioactmat.2023.04.011

49. Olsson R, Carlsson PO. The pancreatic islet endothelial cell: emerging roles in islet function and disease. *Int J Biochem Cell Biol* (2006) 38:492–7. doi: 10.1016/j.biocel.2005.06.021

50. Murakami T, Fujita T. Microcirculation of the rat pancreas, with special reference to the insulo-acinar portal and insulo-venous drainage systems: a further scanning electron microscope study of corrosion casts. *Arch Histol cytology* (1992) 55:453–76. doi: 10.1679/aohc.55.453

51. Dybala MP, Kuznetsov A, Motobu M, Hendren-Santiago BK, Philipson LH, Chervonsky AV, et al. Integrated pancreatic blood flow: bidirectional microcirculation between endocrine and exocrine pancreas. *Diabetes* (2020) 69:1439–50. doi: 10.2337/db19-1034

52. Dybala MP, Gebien LR, Reyna ME, Yu Y, Hara M. Implications of integrated pancreatic microcirculation: crosstalk between endocrine and exocrine compartments. *Diabetes* (2020) 69:2566–74. doi: 10.2337/db20-0810

53. Xu X, Wu L, Lu ZQ, Xia P, Zhu XP, Gao X. Effects of tetramethylpyrazine phosphate on pancreatic islet microcirculation in SD rats. *J endocrinological Invest* (2018) 41:411–9. doi: 10.1007/s40618-017-0748-1

54. Ihoriya C, Satoh M, Kuwabara A, Sasaki T, Kashihara N. Angiotensin II regulates islet microcirculation and insulin secretion in mice. *Microcirculation (New York N.Y.* (2014) 1994) 21:112–23. doi: 10.1111/micc.12094

55. Sakata N, Obenaus A, Chan N, Mace J, Chinnock R, Hathout E. Factors affecting islet graft embolization in the liver of diabetic mice. *Islets* (2009) 1:26–33. doi: 10.4161/isl.1.1.8563

56. Bennet W, Groth CG, Larsson R, Nilsson B, Korsgren O. Isolated human islets trigger an instant blood mediated inflammatory reaction: implications for intraportal islet transplantation as a treatment for patients with type 1 diabetes. *Upsala J Med Sci* (2000) 105:125–33. doi: 10.1517/03009734000000059

57. Moberg L, Johansson H, Lukinius A, Berne C, Foss A, Källen R, et al. Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. *Lancet (London England)* (2002) 360:2039–45. doi: 10.1016/S0140-6736(02)12020-4

58. Hårdstedt M, Lindblom S, Karlsson-Parra A, Nilsson B, Korsgren O. Characterization of innate immunity in an extended whole blood model of human islet allotransplantation. *Cell Transplant* (2016) 25:503–15. doi: 10.3727/096368915X688461

59. Nilsson B, Ekdahl KN, Korsgren O. Control of instant blood-mediated inflammatory reaction to improve islets of Langerhans engraftment. *Curr Opin Organ Transplant* (2011) 16:620–6. doi: 10.1097/MOT.0b013e32834c2393

60. Tjernberg J, Ekdahl KN, Lambris JD, Korsgren O, Nilsson B. Acute antibody-mediated complement activation mediates lysis of pancreatic islets cells and may cause tissue loss in clinical islet transplantation. *Transplantation* (2008) 85:1193–9. doi: 10.1097/TP.0b013e31816b22f3

61. Kanak MA, Takita M, Kunnathodi F, Lawrence MC, Levy MF, Naziruddin B. Inflammatory response in islet transplantation. *Int J Endocrinol* (2014) 2014:451035. doi: 10.1155/2014/451035

62. Marfil-Garza BA, Imes S, Verhoeff K, Hefler J, Lam A, Dajani K, et al. Pancreatic islet transplantation in type 1 diabetes: 20-year experience from a single-centre cohort in Canada. *Lancet Diabetes Endocrinol* (2022) 10:519–32. doi: 10.1016/S2213-8587(22)00114-0

63. Croden J, Silva JR, Huang W, Gupta N, Fu W, Matovinovic K, et al. Cyanidin-3-O-Glucoside improves the viability of human islet cells treated with amylin or Aβ1-42 in vitro. *PloS One* (2021) 16:e0258208. doi: 10.1371/journal.pone.0258208

64. Vítek L, Jirsa M, Brodanová M, Kalab M, Marecek Z, Danzig V, et al. Gilbert syndrome and ischemic heart disease: a protective effect of elevated bilirubin levels. *Atherosclerosis* (2002) 160:449–56. doi: 10.1016/S0021-9150(01)00601-3

65. Vítek L, Novotný L, Sperl M, Holaj R, Spácil J. The inverse association of elevated serum bilirubin levels with subclinical carotid atherosclerosis. *Cerebrovascular Dis (Basel Switzerland)* (2006) 21:408–14. doi: 10.1159/000091966

66. Wiedemann M, Kontush A, Finckh B, Hellwege HH, Kohlschütter A. Neonatal blood plasma is less susceptible to oxidation than adult plasma owing to its higher content of bilirubin and lower content of oxidizable Fatty acids. *Pediatr Res* (2003) 53:843–9. doi: 10.1203/01.PDR.0000057983.95219.0B

67. Sedlak TW, Snyder SH. Bilirubin benefits: cellular protection by a biliverdin reductase antioxidant cycle. *Pediatrics* (2004) 113:1776–82. doi: 10.1542/peds.113.6.1776

68. Fondevila C, Shen XD, Tsuchiyashi S, Yamashita K, Csizmadia E, Lassman C, et al. Biliverdin therapy protects rat livers from ischemia and reperfusion injury. *Hepatol (Baltimore Md.)* (2004) 40:1333–41. doi: 10.1002/hep.20480

69. Lanone S, Bloc S, Foresti R, Almolki A, Taillé C, Callebert J, et al. Bilirubin decreases nos2 expression via inhibition of NAD(P)H oxidase: implications for protection against endotoxic shock in rats. *FASEB journal: Off Publ Fed Am Societies Exp Biol* (2005) 19:1890–2. doi: 10.1096/fj.04-2368fje

70. Nakao A, Neto JS, Kanno S, Stolz DB, Kimizuka K, Liu F, et al. Protection against ischemia/reperfusion injury in cardiac and renal transplantation with carbon monoxide, biliverdin and both. *Am J transplantation: Off J Am Soc Transplant Am Soc Transplant Surgeons* (2005) 5:282–91. doi: 10.1111/j.1600-6143.2004.00695.x

71. Overhaus M, Moore BA, Barbato JE, Behrendt FF, Doering JG, Bauer AJ. Biliverdin protects against polymicrobial sepsis by modulating inflammatory mediators. *Am J Physiol Gastrointestinal liver Physiol* (2006) 290(4):G695–703. doi: 10.1152/ajpgi.00152.2005

72. Li Y, Huang B, Ye T, Wang Y, Xia D, Qian J. Physiological concentrations of bilirubin control inflammatory response by inhibiting NF-κB and inflammasome activation. *Int Immunopharmacol* (2020) 84:106520. doi: 10.1016/j.intimp.2020.106520

73. Mancuso C, Pani G, Calabrese V. Bilirubin: an endogenous scavenger of nitric oxide and reactive nitrogen species. *Redox report: Commun Free Radical Res* (2006) 11:207–13. doi: 10.1179/135100006X154978

74. Idelman G, Smith DLH, Zucker SD. Bilirubin inhibits the up-regulation of inducible nitric oxide synthase by scavenging reactive oxygen species generated by the toll-like receptor 4-dependent activation of NADPH oxidase. *Redox Biol* (2015) 5:398–408. doi: 10.1016/j.redox.2015.06.008

75. Zhu H, Wang J, Jiang H, Ma Y, Pan S, Reddy S, et al. Bilirubin protects grafts against nonspecific inflammation-induced injury in syngeneic intraportal islet transplantation. *Exp Mol Med* (2010) 42:739–48. doi: 10.3858/emm.2010.42.11.075

76. Zhu HQ, Gao Y, Guo HR, Kong QZ, Ma Y, Wang JZ, et al. Pretreatment with bilirubin protects islet against oxidative injury during isolation and purification. *Transplant Proc* (2011) 43:1810–4. doi: 10.1016/j.transproceed.2010.12.058

77. Citro A, Cantarelli E, Pellegrini S, Dugnani E, Piemonti L. Anti-inflammatory strategies in intrahepatic islet transplantation: A comparative study in preclinical models. *Transplantation* (2018) 102:240–8. doi: 10.1097/TP.0000000000001925

78. Citro A, Cantarelli E, Piemonti L. Anti-inflammatory strategies to enhance islet engraftment and survival. *Curr Diabetes Rep* (2013) 13:733–44. doi: 10.1007/s11892-013-0401-0

79. Itoh T, Iwahashi S, Kanak MA, Shimoda M, Takita M, Chujo D, et al. Elevation of high-mobility group box 1 after clinical autologous islet transplantation and its inverse correlation with outcomes. *Cell Transplant* (2014) 23:153–65. doi: 10.3727/096368912X658980

80. Matsuoka N, Itoh T, Watarai H, Sekine-Kondo E, Nagata N, Okamoto K, et al. High-mobility group box 1 is involved in the initial events of early loss of transplanted islets in mice. *J Clin Invest* (2010) 120:735–43. doi: 10.1172/JCI41360

81. Jo EH, Hwang YH, Lee DY. Encapsulation of pancreatic islet with HMGB1 fragment for attenuating inflammation. *Biomaterials Res* (2015) 19:21. doi: 10.1186/s40824-015-0042-2

82. Tokodai K, Goto M, Inagaki A, Nakanishi W, Ogawa N, Satoh K, et al. Attenuation of cross-talk between the complement and coagulation cascades by C5a blockade improves early outcomes after intraportal islet transplantation. *Transplantation* (2010) 90:1358–65. doi: 10.1097/TP.0b013e3181ffb9f5

83. Lundgren T, Bennet W, Tibell A, Söderlund J, Sundberg B, Song Z, et al. Soluble complement receptor 1 (TP10) preserves adult porcine islet morphology after intraportal transplantation into cynomolgus monkeys. *Transplant Proc* (2001) 33:725. doi: 10.1016/S0041-1345(00)02800-1

84. Bennet W, Sundberg B, Lundgren T, Tibell A, Groth CG, Richards A, et al. Damage to porcine islets of Langerhans after exposure to human blood in *vitro*, or after intraportal transplantation to cynomologus monkeys: protective effects of sCR1 and heparin. *Transplantation* (2000) 69:711–9. doi: 10.1097/00007890-200003150-00007

85. Duneton C, Winterberg PD, Ford ML. Activation and regulation of alloreactive T cell immunity in solid organ transplantation. *Nat Rev Nephrol* (2022) 18:663–76. doi: 10.1038/s41581-022-00600-0

86. Halloran PF, Einecke G, Sikosana MLN, Madill-Thomsen K. The biology and molecular basis of organ transplant rejection. *Handb Exp Pharmacol* (2022) 272:1–26. doi: 10.1007/164_2021_557

87. Vilches C, Parham P. Do NK-cell receptors and alloreactivity affect solid organ transplantation? *Transplant Immunol* (2006) 17:27–30. doi: 10.1016/j.trim.2006.09.022

88. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nat Immunol* (2008) 9:503–10. doi: 10.1038/ni1582

89. Li J, Li C, Zhuang Q, Peng B, Zhu Y, Ye Q, et al. The evolving roles of macrophages in organ transplantation. *J Immunol Res* (2019) 2019:5763430. doi: 10.1155/2019/5763430

90. Ronca V, Wootton G, Milani C, Cain O. The immunological basis of liver allograft rejection. *Front Immunol* (2020) 11:2155. doi: 10.3389/fimmu.2020.02155

91. Agarwal D, Allman D, Naji A. Novel therapeutic opportunities afforded by plasma cell biology in transplantation. *Am J transplantation: Off J Am Soc Transplant Am Soc Transplant Surgeons* (2020) 20:1984–91. doi: 10.1111/ajt.15813

92. Yi SG, Gaber AO, Chen W. B-cell response in solid organ transplantation. *Front Immunol* (2022) 13:895157. doi: 10.3389/fimmu.2022.895157

93. Morrell MR, Pilewski JM, Gries CJ, Pipeling MR, Crespo MM, Ensor CR, et al. *De novo* donor-specific HLA antibodies are associated with early and high-grade bronchiolitis obliterans syndrome and death after lung transplantation. *J Heart Lung transplantation: Off Publ Int Soc Heart Transplant* (2014) 33:1288–94. doi: 10.1016/j.healun.2014.07.018

94. Loupy A, Hill GS, Jordan SC. The impact of donor-specific anti-HLA antibodies on late kidney allograft failure. *Nat Rev Nephrol* (2012) 8:348–57. doi: 10.1038/nrneph.2012.81

95. Ho EK, Vlad G, Vasilescu ER, de la Torre L, Colovai AI, Burke E, et al. Pre- and posttransplantation allosensitization in heart allograft recipients: major impact of *de*

novo alloantibody production on allograft survival. *Hum Immunol* (2011) 72:5–10. doi: 10.1016/j.humimm.2010.10.013

96. Kanamori M, Nakatsukasa H, Okada M, Lu Q, Yoshimura A. Induced regulatory T cells: their development, stability, and applications. *Trends Immunol* (2016) 37:803–11. doi: 10.1016/j.it.2016.08.012

97. Alhabbab RY, Nova-Lamperti E, Aravena O, Burton HM, Lechler RI, Dorling A, et al. Regulatory B cells: Development, phenotypes, functions, and role in transplantation. *Immunol Rev* (2019) 292:164–79. doi: 10.1111/imr.12800

98. Wood KJ, Bushell A, Hester J. Regulatory immune cells in transplantation. *Nat Rev Immunol* (2012) 12:417–30. doi: 10.1038/nri3227

99. Plenter RJ, Grazia TJ, Doan AN, Gill RG, Pietra BA. CD4 T cells mediate cardiac xenograft rejection via host MHC Class II. *J Heart Lung transplantation: Off Publ Int Soc Heart Transplant* (2012) 31:1018–24. doi: 10.1016/j.healun.2012.05.018

100. Le Moine A, Goldman M. Non-classical pathways of cell-mediated allograft rejection: new challenges for tolerance induction? *Am J transplantation: Off J Am Soc Transplant Am Soc Transplant Surgeons* (2003) 3:101–6. doi: 10.1034/j.1600-6143.2002.00026.x

101. Landstra CP, Nijhoff MF, Roelen DL, de Vries APJ, de Koning EJP. Diagnosis and treatment of allograft rejection in islet transplantation. *Am J transplantation: Off J Am Soc Transplant Am Soc Transplant Surgeons* (2023) 23:1425–33. doi: 10.1016/j.ajt.2023.05.035

102. Pardoll DM. Distinct mechanisms of tumor resistance to NK killing: of mice and men. *Immunity* (2015) 42:605–6. doi: 10.1016/j.immuni.2015.04.007

103. Shi Y, Zhao YZ, Jiang Z, Wang Z, Wang Q, Kou L, et al. Immune-protective formulations and process strategies for improved survival and function of transplanted islets. *Front Immunol* (2022) 13:923241. doi: 10.3389/fimmu.2022.923241

104. DeWolf S, Sykes M. Alloimmune T cells in transplantation. *J Clin Invest* (2017) 127:2473–81. doi: 10.1172/JCI90595

105. Grewal IS, Flavell RA. The role of CD40 ligand in costimulation and T-cell activation. *Immunol Rev* (1996) 153:85–106. doi: 10.1111/j.1600-065X.1996.tb00921.x

106. Pishesha N, Harmand TJ, Ploegh HL. A guide to antigen processing and presentation. *Nat Rev Immunol* (2022) 22:751–64. doi: 10.1038/s41577-022-00707-2

107. Montano-Loza AJ, Rodríguez-Perálvarez ML, Pageaux GP, Sanchez-Fueyo A, Feng S. Liver transplantation immunology: Immunosuppression, rejection, and immunomodulation. *J Hepatol* (2023) 78:1199–215. doi: 10.1016/j.jhep.2023.01.030

108. Barra JM, Tse HM. Redox-dependent inflammation in islet transplantation rejection. *Front Endocrinol* (2018) 9:175. doi: 10.3389/fendo.2018.00175

109. Grinyó JM, Cruzado JM. Mycophenolate mofetil and calcineurin-inhibitor reduction: recent progress. *Am J transplantation: Off J Am Soc Transplant Am Soc Transplant Surgeons* (2009) 9:2447–52. doi: 10.1111/j.1600-6143.2009.02812.x

110. Kepple JD, Barra JM, Young ME, Hunter CS, Tse HM. Islet transplantation into brown adipose tissue can delay immune rejection. *JCI Insight* (2022) 7. doi: 10.1172/jci.insight.152800

111. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *New Engl J Med* (2000) 343:230–8. doi: 10.1056/NEJM200007273430401

112. Nanji SA, Shapiro AM. Islet transplantation in patients with diabetes mellitus: choice of immunosuppression. *BioDrugs: Clin immunotherapeutics biopharmaceuticals Gene Ther* (2004) 18:315–28. doi: 10.2165/00063030-200418050-00004

113. Roep BO, Stobbe I, Duinkerken G, van Rood JJ, Lernmark A, Keymeulen B, et al. Auto- and alloimmune reactivity to human islet allografts transplanted into type 1 diabetic patients. *Diabetes* (1999) 48:484–90. doi: 10.2337/diabetes.48.3.484

114. Shapiro AM, Gallant HL, Hao EG, Lakey JR, McCready T, Rajotte RV, et al. The portal immunosuppressive storm: relevance to islet transplantation? *Ther Drug Monit* (2005) 27:35–7. doi: 10.1097/00007691-200502000-00008

115. Knechtle SJ, Vargo D, Fechner J, Zhai Y, Wang J, Hanaway MJ, et al. FN18-CRM9 immunotoxin promotes tolerance in primate renal allografts. *Transplantation* (1997) 63:1–6. doi: 10.1097/00007890-199701150-00002

116. Coronel MM, Linderman SW, Martin KE, Hunckler MD, Medina JD, Barber G, et al. Delayed graft rejection in autoimmune islet transplantation via biomaterial immunotherapy. *Am J transplantation: Off J Am Soc Transplant Am Soc Transplant Surgeons* (2023) 23:1709–22. doi: 10.1016/j.ajt.2023.07.023

117. Crisa L, Cirulli V. Bone marrow chimerism breaks the barrier to pancreatic islet transplantation. *Cell Rep* (2022) 41:111692. doi: 10.1016/j.celrep.2022.111692

118. Sykes M. Mixed chimerism and transplant tolerance. *Immunity* (2001) 14:417–24. doi: 10.1016/S1074-7613(01)00122-4

119. Feuerer M, Hill JA, Mathis D, Benoist C. Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. *Nat Immunol* (2009) 10:689–95. doi: 10.1038/ni.1760

120. Gerace D, Zhou Q, Kenty JH, Veres A, Sintov E, Wang X, et al. Engineering human stem cell-derived islets to evade immune rejection and promote localized immune tolerance. *Cell Rep Med* (2023) 4:100879. doi: 10.1016/j.xcrm.2022.100879

121. Vågesjö E, Christoffersson G, Waldén TB, Carlsson PO, Essand M, Korsgren O, et al. Immunological shielding by induced recruitment of regulatory T-lymphocytes delays rejection of islets transplanted in muscle. *Cell Transplant* (2015) 24:263–76. doi: 10.3727/096368914X678535

122. Li Y, Frei AW, Labrada IM, Rong Y, Liang JP, Samojlik MM, et al. Immunosuppressive PLGA TGF-β1 microparticles induce polyclonal and antigen-specific regulatory T cells for local immunomodulation of allogeneic islet transplants. *Front Immunol* (2021) 12:653088. doi: 10.3389/fimmu.2021.653088

123. Stucchi A, Maspes F, Montee-Rodrigues E, Fousteri G. Engineered Treg cells: The heir to the throne of immunotherapy. *J Autoimmun* (2023) 102986. doi: 10.1016/j.jaut.2022.102986

124. Boardman DA, Philippeos C, Fruhwirth GO, Ibrahim MA, Hannen RF, Cooper D, et al. Expression of a chimeric antigen receptor specific for donor HLA class I enhances the potency of human regulatory T cells in preventing human skin transplant rejection. *Am J transplantation: Off J Am Soc Transplant Am Soc Transplant Surgeons* (2017) 17:931–43. doi: 10.1111/ajt.14185

125. Radichev IA, Yoon J, Scott DW, Griffin K, Savinov AY. Towards antigen-specific Tregs for type 1 diabetes: Construction and functional assessment of pancreatic endocrine marker, HPi2-based chimeric antigen receptor. *Cell Immunol* (2020) 358:104224. doi: 10.1016/j.cellimm.2020.104224

126. Dawson NAJ, Rosado-Sánchez I, Novakovsky GE, Fung VCW, Huang Q, McIver E, et al. Functional effects of chimeric antigen receptor co-receptor signaling domains in human regulatory T cells. *Sci Trans Med* (2020) 12. doi: 10.1126/scitranslmed.aaz3866

127. Blat D, Zigmond E, Alteber Z, Waks T, Eshhar Z. Suppression of murine colitis and its associated cancer by carcinoembryonic antigen-specific regulatory T cells. *Mol therapy: J Am Soc Gene Ther* (2014) 22:1018–28. doi: 10.1038/mt.2014.41

128. Yoon J, Schmidt A, Zhang AH, Königs C, Kim YC, Scott DW. FVIII-specific human chimeric antigen receptor T-regulatory cells suppress T- and B-cell responses to FVIII. *Blood* (2017) 129:238–45. doi: 10.1182/blood-2016-07-727834

129. Zafar A, Lee J, Yesmin S, Paget MB, Bailey CJ, Murray HE, et al. Rotational culture and integration with amniotic stem cells reduce porcine islet immunoreactivity in *vitro* and slow xeno-rejection in a murine model of islet transplantation. *Xenotransplantation* (2019) 26:e12508. doi: 10.1111/xen.12508

130. Jun Y, Kim MJ, Hwang YH, Jeon EA, Kang AR, Lee SH, et al. Microfluidics-generated pancreatic islet microfibers for enhanced immunoprotection. *Biomaterials* (2013) 34:8122–30. doi: 10.1016/j.biomaterials.2013.07.079

131. Su J, Hu BH, Lowe WL Jr., Kaufman DB, Messersmith PB. Anti-inflammatory peptide-functionalized hydrogels for insulin-secreting cell encapsulation. *Biomaterials* (2010) 31:308–14. doi: 10.1016/j.biomaterials.2009.09.045

132. Peeters LEJ, Andrews LM, Hesselink DA, de Winter BCM, van Gelder T. Personalized immunosuppression in elderly renal transplant recipients. *Pharmacol Res* (2018) 130:303–7. doi: 10.1016/j.phrs.2018.02.031

133. Brunet M, Shipkova M, van Gelder T, Wieland E, Sommerer C, Budde K, et al. Barcelona consensus on biomarker-based immunosuppressive drugs management in solid organ transplantation. *Ther Drug Monit* (2016) 38 Suppl 1:S1–20. doi: 10.1097/FTD.0000000000000287

134. Olbricht CJ. Why do we need biomarkers in solid organ transplantation. *Clinica chimica acta; Int J Clin Chem* (2012) 413:1310–1. doi: 10.1016/j.cca.2012.04.026

135. Anglicheau D, Naesens M, Essig M, Gwinner W, Marquet P. Establishing biomarkers in transplant medicine: A critical review of current approaches. *Transplantation* (2016) 100:2024–38. doi: 10.1097/TP.0000000000001321

136. Merola J, Emond JC, Levitsky J. Novel noninvasive biomarkers in liver transplantation: A tool on the doorstep of clinical utilization. *Transplantation* (2023) 107:2120–5. doi: 10.1097/TP.0000000000004580

137. Beckmann JH, Heits N, Braun F, Becker T. [Immunological markers in organ transplantation]. *Zentralblatt fur Chirurgie* (2017) 142:161–8. doi: 10.1055/s-0032-1328352

138. Coronel MM, Martin KE, Hunckler MD, Barber G, O'Neill EB, Medina JD, et al. Immunotherapy via PD-L1-presenting biomaterials leads to long-term islet graft survival. *Sci Adv* (2020) 6:eaba5573. doi: 10.1126/sciadv.aba5573

139. Yang J, Popoola J, Khandwala S, Vadivel N, Vanguri V, Yuan X, et al. Critical role of donor tissue expression of programmed death ligand-1 in regulating cardiac allograft rejection and vasculopathy. *Circulation* (2008) 117:660–9. doi: 10.1161/CIRCULATIONAHA.107.741025

140. Yoshihara E, O'Connor C, Gasser E, Wei Z, Oh TG, Tseng TW, et al. Immune-evasive human islet-like organoids ameliorate diabetes. *Nature* (2020) 586:606–11. doi: 10.1038/s41586-020-2631-z

141. Navarro-Alvarez N, Yang YG. CD47: a new player in phagocytosis and xenograft rejection. *Cell Mol Immunol* (2011) 8:285–8. doi: 10.1038/cmi.2010.83

142. Finley MJ, Rauova L, Alferiev IS, Weisel JW, Levy RJ, Stachelek SJ. Diminished adhesion and activation of platelets and neutrophils with CD47 functionalized blood contacting surfaces. *Biomaterials* (2012) 33:5803–11. doi: 10.1016/j.biomaterials.2012.04.051

143. Chhabra A, Ring AM, Weiskopf K, Schnorr PJ, Gordon S, Le AC, et al. Hematopoietic stem cell transplantation in immunocompetent hosts without radiation or chemotherapy. *Sci Trans Med* (2016) 8(351):351ra105. doi: 10.1126/scitranslmed.aae0501

144. Demeure CE, Tanaka H, Mateo V, Rubio M, Delespesse G, Sarfati M. CD47 engagement inhibits cytokine production and maturation of human dendritic cells. *J Immunol (Baltimore Md.* (2000) 1950) 164:2193–9. doi: 10.4049/jimmunol.164.4.2193

145. Latour S, Tanaka H, Demeure C, Mateo V, Rubio M, Brown EJ, et al. Bidirectional negative regulation of human T and dendritic cells by CD47 and its

cognate receptor signal-regulator protein-alpha: down-regulation of IL-12 responsiveness and inhibition of dendritic cell activation. *J Immunol (Baltimore Md.* (2001) 1950) 167:2547–54. doi: 10.4049/jimmunol.167.5.2547

146. Shrestha P, Batra L, Tariq Malik M, Tan M, Yolcu ES, Shirwan H. Immune checkpoint CD47 molecule engineered islets mitigate instant blood-mediated inflammatory reaction and show improved engraftment following intraportal transplantation. *Am J transplantation: Off J Am Soc Transplant Am Soc Transplant Surgeons* (2020) 20:2703–14. doi: 10.1111/ajt.15958

147. Ariyan C, Salvalaggio P, Fecteau S, Deng S, Rogozinski L, Mandelbrot D, et al. Cutting edge: transplantation tolerance through enhanced CTLA-4 expression. *J Immunol (Baltimore Md.* (2003) 1950) 171:5673–7. doi: 10.4049/jimmunol.171.11.5673

148. Zhang W, Gorantla VS, Campbell PG, Li Y, Yang Y, Komatsu C, et al. Biopatterned CTLA4/fc matrices facilitate local immunomodulation, engraftment, and glucose homeostasis after pancreatic islet transplantation. *Diabetes* (2016) 65:3660–6. doi: 10.2337/db16-0320

149. Yamada A, Arakaki R, Saito M, Kudo Y, Ishimaru N. Dual role of fas/fasL-mediated signal in peripheral immune tolerance. *Front Immunol* (2017) 8:403. doi: 10.3389/fimmu.2017.00403

150. Kurts C, Heath WR, Kosaka H, Miller JF, Carbone FR. The peripheral deletion of autoreactive CD8+ T cells induced by cross-presentation of self-antigens involves signaling through CD95 (Fas, Apo-1). *J Exp Med* (1998) 188:415–20. doi: 10.1084/jem.188.2.415

151. Stranges PB, Watson J, Cooper CJ, Choisy-Rossi CM, Stonebraker AC, Beighton RA, et al. Elimination of antigen-presenting cells and autoreactive T cells by Fas contributes to prevention of autoimmunity. *Immunity* (2007) 26:629–41. doi: 10.1016/j.immuni.2007.03.016

152. Bleesing JJ, Straus SE, Fleisher TA. Autoimmune lymphoproliferative syndrome. *A Hum Disord Abnormal lymphocyte survival. Pediatr Clinics North America* (2000) 47:1291–310. doi: 10.1016/S0031-3955(05)70272-8

153. Skoumal M, Woodward KB, Zhao H, Wang F, Yolcu ES, Pearson RM, et al. Localized immune tolerance from FasL-functionalized PLG scaffolds. *Biomaterials* (2019) 192:271–81. doi: 10.1016/j.biomaterials.2018.11.015

154. Merani S, Toso C, Emamaullee J, Shapiro AM. Optimal implantation site for pancreatic islet transplantation. *Br J Surg* (2008) 95:1449–61. doi: 10.1002/bjs.6391

155. Van Der Windt DJ, Echeverri GJ, Ijzermans JNM, Cooper DKC. The choice of anatomical site for islet transplantation. *Cell Transplant* (2008) 17:1005–14. doi: 10.3727/096368908786991515

156. Smood B, Bottino R, Hara H, Cooper DKC. Is the renal subcapsular space the preferred site for clinical porcine islet xenotransplantation? *Rev article. Int J Surg (London England)* (2019) 69:100–7. doi: 10.1016/j.ijsu.2019.07.032

157. Sakata N, Aoki T, Yoshimatsu G, Tsuchiya H, Hata T, Katayose Y, et al. Strategy for clinical setting in intramuscular and subcutaneous islet transplantation. *Diabetes/metabolism Res Rev* (2014) 30:1–10. doi: 10.1002/dmrr.2463

158. Weaver JD, Headen DM, Aquart J, Johnson CT, Shea LD, Shirwan H, et al. Vasculogenic hydrogel enhances islet survival, engraftment, and function in leading extrahepatic sites. *Sci Adv* (2017) 3:e1700184. doi: 10.1126/sciadv.1700184

159. Sakata N, Yoshimatsu G, Kodama S. The spleen as an optimal site for islet transplantation and a source of mesenchymal stem cells. *Int J Mol Sci* (2018) 19. doi: 10.20944/preprints201802.0101.v2

160. Liang Z, Sun D, Lu S, Lei Z, Wang S, Luo Z, et al. Implantation underneath the abdominal anterior rectus sheath enables effective and functional engraftment of stem-cell-derived islets. *Nat Metab* (2023) 5:29–40. doi: 10.1038/s42255-022-00713-7

frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 11 January 2024
DOI 10.3389/fimmu.2023.1242478

OPEN ACCESS

EDITED BY
Lisha Mou,
Shenzhen Second People's Hospital, China

REVIEWED BY
Pradeep Shrestha,
University of Texas MD Anderson Cancer Center, United States

*CORRESPONDENCE
Xiaozhou He
hyx@suda.edu.cn

RECEIVED 19 June 2023
ACCEPTED 13 December 2023
PUBLISHED 11 January 2024

CITATION
Xu H and He X (2024) Developments in kidney xenotransplantation.
Front. Immunol. 14:1242478.
doi: 10.3389/fimmu.2023.1242478

Developments in kidney xenotransplantation

Haiyan Xu and Xiaozhou He*

Urology Department, Third Affiliated Hospital of Soochow University, Changzhou, China

The search for kidney xenografts that are appropriate for patients with end-stage renal disease has been ongoing since the beginning of the last century. The major cause of xenograft loss is hyperacute and acute rejection, and this has almost been overcome via scientific progress. The success of two pre-clinical trials of α1,3-galactosyltransferase gene-knockout porcine kidneys in brain-dead patients in 2021 triggered research enthusiasm for kidney xenotransplantation. This minireview summarizes key issues from an immunological perspective: the discovery of key xenoantigens, investigations into key co-stimulatory signal inhibition, gene-editing technology, and immune tolerance induction. Further developments in immunology, particularly immunometabolism, might help promote the long-term outcomes of kidney xenografts.

KEYWORDS

kidney xenotransplantation, α-Gal, CD40L-CD40, gene editing, tolerance induction

1 Introduction

Xenotransplantation can play key roles in reducing the kidney donor shortage. Since the first kidney xenotransplant in 1906 (1), great strides have led to achievements in xenotransplantation such that the risk of hyperacute and acute rejection is almost overcome (2, 3). Significant progress has been made in key issues in xenotransplantation (4–6). Important events in kidney xenotransplantation and the advancements of immunological theories and techniques in corresponding periods are listed in Figure 1.

Here we especially discuss the pivotal developments of kidney xenotransplantation from an immunological perspective (Table 1).

2 Key xenoantigens

The recognition of xenoantigens involved in hyperacute rejection has been a long and tortuous road. The first interzygotic twin transplantation in 1953 resulted in long recipient survival and revealed a new direction for organ transplantation. With the discovery and application of immunosuppressive agents, hyperacute rejection after allotransplantation could be controlled, and the survival of recipients gradually increased. However, hyperacute

FIGURE 1
Timeline of developments in kidney xenotransplantation 1906-2022.

rejection after xenotransplantation cannot be controlled by the empirical application of immunosuppressive agents (7).

Recipient rabbits treated with homogenized guinea pig liver mixtures survived longer after guinea pig kidney grafts were transplanted (8). This inspired many attempts to reduce hyperacute rejection of xenografts, such as the selective removal of plasma components (9), elimination of extant antibodies, inhibition of coagulation, as well as the synthesis of complement and antibodies (10). The results suggested that hyperacute rejection of xenografts is strongly associated with donor antigens, plasma composition, and antibody synthesis, similar to hyperacute rejection during allotransplantation.

2.1 α-Gal antigen

The red blood cell surface galactose antigen (DGalα1→3DGal) that induces hyperacute homotransplant rejection due to an ABO mismatch was identified in the late 1980s (11). During xenotransplantation, hyperacute rejection results in an abnormal increase in immunoglobulin (Ig)M serum levels rather than in IgG levels. This indicates that the recipient's immune system first recognizes the specific antigens harbored in xenografts.

Due to the emergence of monoclonal antibodies (mAbs) using hybridomas, human anti-swine antibodies waere generated and used to identify significant carbohydrate structures for xenotransplantation (12). Then the α-galactosyltransferase (α-Gal) was found, which is encoded by the α-1,3-galactosyltransferase (*GGTA1*) gene (13). Other carbohydrate antigens, such as non-fucosylated chondroitin sulfate monolayers and linear antigens, are also found, locating on the surfaces of all porcine vascular endothelial cells. These antigens tightly bind to anti-Gal isogalectin β4 antibodies and specifically bind to natural, human anti-α-Gal antibodies. Gal epitopes are expressed abundantly in the brush margins of proximal convoluted tubules, moderately in distal convoluted tubules, and not at all in renal collecting tubules and glomeruli. A specific antigen-antibody reaction activates the complement system, leading to a powerful cytotoxic effect that leads to hyperacute grafts (14–18). The discovery of the α-Gal antigen was a major breakthrough in xenotransplantation.

Thereafter, considerable efforts were directed toward decreasing hyperacute rejection of kidney xenotransplants by removing anti-

TABLE 1 Critical progress in promoting kidney xenograft survival.

Discovery of xenoantigens	
Carbohydrate antigen	α-Gal
	Non-α-Gal
Proteantigen	SLA
Investigation into key co-stimulatory signal pathways	
Anti-CD40/anti-CD40L	
Establishment of genetically engineered pigs	
CRISPR/Cas9//Human CD55, CD59, CD46, CD39	
Immune tolerance induction by chimerism	
"Thymus kidney"	
Bone marrow/Hematopoietic cells	
Research interests	

porcine antibodies *in vitro*, short-term infusions of specific carbohydrates (19), or the absorption of anti-xenoantigen antibodies produced in the spleen and kidneys (20). Soluble Gal proteins can partially inhibit human rejection of porcine kidneys. Intravenous infusions of bovine serum albumin-Gal *in vivo* can essentially maintain the depletion of circulating anti-Gal antibodies and prevent or delay antibody deposition and the acute humoral rejection of pig-to-baboon xenografts, but it might be associated with liver damage (21).

2.2 Non-α-galantigens (Neu5Gc, CMAH and B4GalNT2)

Transgenic technology was established in 1981 using microinjections; and a transgenic mouse model was created in 1982. The first generation of the gene-editing tool, zinc finger nuclease, was introduced during the late 1990s, and another, transcriptional activator-like effector nuclease, was identified in 2009. These gene-editing techniques had a positive global impact on life sciences.

Pigs with α-Gal knockout (α-Gal$^{-/-}$, GTKO) are important xenotransplantation models (22–24). In the α-Gal$^{-/-}$ pigs to baboon kidney xenotransplantation models, most recipients did not develop hyperacute rejection; however, they succumbed to acute humoral rejection. The significantly increased abundance of peripheral anti-non-α-Gal antibodies in recipients suggested that non-α-Gal antigens in kidney xenografts might trigger the production of large amounts of corresponding antibodies. Thereafter, non-α-Gal antigens were recognized as obstacles to α-Gal$^{-/-}$ pig organ xenotransplantation (25). The α-Gal antigen is crucial for hyperacute rejection, and non-α-Gal antigens play important roles in humoral rejection of xenotransplants. In addition to α-Gal and non-α-Gal, other carbohydrate antigens have a complex spatial distribution in porcine kidneys and are strongly associated with the outcome of porcine kidney xenotransplantation (26).

Non-α-Gal antigens, such as N-glycolylneuraminic acid (Neu5Gc; HD antigen), encoded by the cytidine monophospho-N-acetylneuraminic acid hydroxylase (*cMAH*) gene have been identified (27, 28). Compared with GGTA1$^{-/-}$ pig xenotransplantation, humoral rejection is reduced in GGTA1$^{-/-}$/CMAH$^{-/-}$ pigs xenotransplantation (29), implying that the immune heritability of the Neu5Gc antigen potentially plays an important role in pig-human xenotransplantation. The other carbohydrate non-α-Gal antigen, glycosyltransferase, (SD(a) antigen), is encoded by the β-1,4-N-acetyl-galactosaminyl transferase (*B4GalNT2*) gene (30, 31).

Clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (Cas9) is a third-generation gene-editing tool. Porcine embryonic fibroblasts with GGTA1$^{-/-}$/Gal$^{-/-}$ were initially created using CRISPR/Cas9 in 2014 (32). Since then, CRISPR/Cas9 has become the preferred means of generating genetically engineered pigs. The serum of many waitlisted patients contained only a minimal number of antibodies that reacted with peripheral blood mononuclear cells from GGTA1$^{-/-}$/CMAH$^{-/-}$/B4GalNT2$^{-/-}$ pigs. However, anti-human leukocyte antigen antibodies in some sensitized patients cross-reacted with porcine major histocompatibility complex (MHC) I antibodies (33). Pigs with simultaneous MHC and three antigen (GGTA1/CMAH/B4GalNT2) inactivation have been generated using the CRISPR/Cas method (34). Natural and inducible anti-SDa plays important roles in GTKO pig-to-rhesus monkey xenotransplant rejection, thus providing further support for the notion that Gal and SDa antigens should be simultaneously targeted (35). Exploration of new key non-α-Gal antigens is currently underway (36).

2.3 SLAs

SLAs are being discovered to play an important role in swine innate and adoptive immune responses. In some sensitized kidney transplant-waitlisted patients, some human leucocyte antigen (HLA) antibodies cross-react with SLA class I (37). SLA II is also a xenoantigen (38, 39). And triple (GGTA1, CMAH, B2M) genes modified pigs expressed the SLA I^{low} phenotype, which effects on immune status and susceptibility to human immune responses (40). *In vitro* human TNF-α could increase SLA I expression, while human IL-17 could decrease TNF-α-mediated SLA-I upregulation (41), and downregulation of SLA expression decreases the strength of xenogeneic immune responses towards renal tubular epithelial cells (42). These data may support the SLA-silencing strategy application to prevent xenogeneic cellular immune responses.

3 Blocking CD40L-CD40 co-stimulatory signals

Diversity and specificity of immunoglobulins suggests that cellular and humoral immune responses are not separate entities, but complementary components. T and B lymphocytes interact

to activate and differentiate into effector cells under specific circumstances. During this process, co-stimulatory signals, such as cluster of differentiation (CD) 40 and its ligand CD40L, CD28-B7, and inducible T cell co-stimulator ligand (ICOS) and its ligand ICOSL, play indispensable roles, and the effects of CD40L-CD40 signaling on xenotransplantation have been extensively investigated.

The 35 kDa polypeptide CD40 is mainly expressed in B lymphocytes (43, 44). After CD40L was identified (44–46), numerous *in vivo* and *in vitro* findings showed that the CD40L-CD40 pathway is essential for T cell responses and specific antibody production by B lymphocytes (47–52). The biological effects of anti-CD40L mAb, as well as other related mAbs, including anti-CD80, anti-CD86 mAbs, and biologicals, such as hCTLA4-Ig, have been extensively studied *in vitro* and *in vivo* (53–55). Results suggest that blocking the CD40-CD40L pathway, or combined blocking of the CD28-B7 signal could effectively inhibit T cell activation and suppress the production of specific antibodies.

Data from pig to non-human primates (NHPs) organ xenotransplants reveal that anti-CD40L mAb suppresses CD40-CD40L co-stimulatory signals and decreases T cell-mediated immune responses, whereas natural anti-Gal antibodies are detectable at baseline (56). The application of anti-CD40L mAbs to NHPs is safe (57, 58) and blocking the CD40L-CD40 signal might induce immune nonresponse to a xenotransplant (59, 60); thus, prolonging xenograft survival (61–64). By comparison, co-stimulation blockades with an anti-CD40L agent is more successful than with an anti-CD40 agent (65–67).

Currently, the immunosuppressive regimen based on the blockade of the CD40-CD40L co-stimulation pathway is considered as an extremely important development in the xenotransplantation. As a biological agent, the affinity and effective doses of these mAbs for individuals, the mechanism of action, and the potential side effects, require further investigation.

4 Genetically engineered pig establishment

Expression of the end-stage complement suppressor human CD59 seems to promote the survival of transplanted organs *in vitro* (68, 69). The complement protein CD55 (decay acceleration factor) regulates complements, whereas CD46 is an inhibitory regulator of the complement system. Knocking human CD55, CD59, and CD46 into the pig genomes resulted in their expression in vascular endothelial cells and suppressed damage caused by complement activation (70). Cynomolgus monkeys that received GGTA1$^{-/-}$/CD55 transgene (Tg) pig kidneys survived for >90 days (71), which was surprising at the time. This also suggested that human CD55 knock-in promotes xenograft survival, in addition to preventing ureteral stenosis. Recipient rhesus monkeys with low levels of anti-pig antibodies were screened as recipients of GTKO/human CD55 Tg pigs' kidneys, and the anti-CD40L mAbs applied after transplantation and conventional immunosuppressive protocol resulted in the recipients surviving for >125 days (72).

Thrombomodulin, endothelial protein C receptors, CD39, and other factors function in the regulation of human coagulation. Thrombomodulin and CD39 are involved in complement activation and the coagulation cascade during heterogeneous immune regulation (73–75). In the GTKO/human CD46, CD55, thrombomodulin, endothelial protein C receptors, and CD39 Tg porcine to baboon kidney xenotransplantation models, recipients who received anti-thymocyte globulin (ATG) and anti-CD20 mAb induction, along with anti-CD40 mAb-based immunosuppression therapy survived for up to 136 days (76). In the GTKO/human CD55 Tg porcine to rhesus monkey kidney xenotransplantation models, rhesus monkeys with low antibody titers were selected, some who received transient pan-T cell destruction and the anti-CD40L mAb-based immunotherapy protocol survived for 405 days (77).

The obtained experience in kidney xenotransplantation of genetically engineered pigs to NHPs has provided a solid foundation for pre-clinical trials. The surgeries, α-Gal knockout pigs to brain-dead patient kidney xenotransplantation, were conducted in the USA in 2021, and the survival of xenografts was 54 (2) and 74 (3) h.

5 Tolerance induction by chimerism

5.1 Thymus co-transplantation

Attempts to induce immune tolerance in xenografts by multiple low-dose xenoantigen inoculations have been unsuccessful. Transplanting fetal porcine thymus and liver tissues into mice to eliminate T and natural killer cells and removing the thymus induces specific tolerance to porcine antigens (78). The mouse CD4$^+$ T cell repertoire developed in implanted pig thymus grafts indicated positive selection by porcine (xenogeneic) MHC antigens and negative selection by both mice (recipients) and porcine MHC; this suggested a high level of tolerant immunocompetence (79–81). Findings of kidney allotransplantation in large animals have indicated that the thymus is essential for rapid and stable immune tolerance (82, 83), implying the potential value of thymus transplants to induce tolerance.

The "thymus kidney" was invented by placing thymus tissues under a kidney quilt to facilitate autologous thymus transplantation. The results suggested that the abundance of peripheral CD4$^+$CD45RA$^+$ T cells increased steadily from 30 to 150 days after transplanting "thymus kidneys" into athymic micropigs, and recipient pigs had acquired immune tolerance. Vascularized donor thymus tissue can induce rapid and stable immune tolerance in recipients to MHC-unmatched allograft (84–86).

In "thymus kidneys" xenotransplantation models, recipient baboons transplanted with a "thymus kidney" graft from a human CD55 Tg pig survived for 30 days, and live thymic epithelial cells and thymic bodies, including a few baboon lymphocytes, were discovered under the renal capsule and omentum of the baboons. The "thymus kidney" can induce the production of non-responsive donor-specific cells and stable amounts of anti-α-Gal antibodies, thus inducing immune

tolerance across the genetic immune barrier (87). Transplanting GTKO pig kidneys with the vascular thymus into baboons significantly extended recipients' survival (88). Recipient baboons with or without cortisol transplanted with "thymus kidneys" from GTKO micropigs survived for >80 days with no signs of cellular rejection or IgG deposition in the transplants and no loss of the transplanted kidneys, suggesting establishment of donor-specific T cell tolerance (89).

Fetal porcine thymus grafts containing mice thymic epithelial cells implanted into mice improved the development of T cells in the thymus, increased the likelihood that they would develop tolerance to the grafts, and reconstructed the T cell population (90). The method for preparing donor thymus grafts enriched with recipient thymic epithelial cells in large animals (cynomolgus monkeys and micropigs) was established (91). This should induce the tolerance of transplanted solid organs, including the kidneys (92).

Mouse T cell receptor–transgenic T cells can be functionally educated using porcine MHC antigens (93). Human T cells develop normally in porcine thymus grafts and form specific tolerance to porcine MHC in immunodeficient mice (94). However, a mouse with a transplanted porcine thymus would develop analogous autoimmune diseases, in which mouse $CD4^+$ T cells play a key role (95). Therefore, the differentiation of host T precursor cells in the porcine thymus should differ from the normal physiological state. The number of Tregs in the athymic mice that were grafted with porcine thymus was close to normal, but the regulatory function was not (96). Moreover, T cell differentiation in humanized mice after bone marrow (BM) transplantation revealed that the positive selection was inadequate (97).

These findings should be helpful for thymus transplantation in large animals. Autologous thymus tissues were co-transplanted with GTKO porcine kidneys in the clinical trial of transplantation in two brain-dead patients (2). The results exceeded expectations; however, the mechanisms of tolerance induction need to be further explored.

5.2 BM or hematopoietic cell co-transplantation

Transplanted BM or hematopoietic cells can establish chimera-induced tolerance (98). Long-term survival has been achieved using kidneys co-transplanted with BM (99). Moreover, the role of $CD4^+CD25^+FoxP3^+$Treg cells in these results cannot be ignored (100–102).

Simon et al. (103) injected large doses of porcine spleen cells into baboons and found that low-level chimera status was maintained for almost 1.5 years, during which the baboons did not get sick. These results suggested that donor leukocyte infusion can be used to induce peripheral tolerance during xenotransplantation. Perhaps infusing BM cells with differentiation potential would be more advantageous for establishing chimera-induced immune tolerance.

Griesemer et al. found that baboons transplanted with GTKO BM alone *in vivo* developed peripheral chimeras within 28 days, and the abundance of anti-GTKO porcine antibody or porcine-specific cytotoxicity did not increase. However, anti-porcine and other specific antibodies appeared 14 days after transplantation in baboons that were co-transplanted with BM cells and kidneys, and relatively high levels of anti-Gal antibodies were detected when the porcine kidney was rejected (104). These data suggested that BM infusion is associated with a loss of anti-Gal antibodies. To improve chimerism, the infusion method was modified, and the results were successful, the donor pig kidneys in the two groups survived for 47 and 60 days, respectively (105).

The cell- and species-specific CD47/Signal regulatory protein α (Sirp-α) signaling pathway might be involved in clearing cells derived from porcine BM cells in recipients. Porcine BM transferred the human CD47 gene survived much longer in a recipient baboon, and the chimeras prolonged the survival of porcine skin grafts (106).

6 Research interests

In the past decades, many solutions have been applied to solve the ethics and theoretical issues in kidney xenotransplantation, and the breakthrough achieved are encouraging. In addition to immunology-related issues, the transmission of porcine xenotransplantation-relevant viruses (such as porcine endogenous retroviruses, PERV) were well controlled (107). However, whether PERV remains inactivated depends on the stability of porcine genomes after modified by CRISPR/Cas 9 technique.

Comprehensive analysis suggested that, these following issues should be studied in deepth for a better survival of kidney xenografts.

6.1 Gene-editing techniques should be perfected

Although CRISPR/Cas9 technology is widely applied, it has some limitations, such as off-target effects, low delivery efficiency, and the immune heritability of Cas9 protein. Any unexpected changes in the human (or xenograft) genome could result in serious and unintended consequences, including the activation of proto-oncogenes and production of new single nucleotide polymorphisms that can alter cellular behavior. In addition, >60% of the population harbors components of humoral and cellular immune responses to Cas9. Therefore, if sustained, Cas9 expression is required during treatment and the immune response induced by the Cas9 must be considered (108). Improvements in CRISPR/Cas9 technology will be conducive to the long-term outcome of clinical kidney xenotransplantation (109).

6.2 Deeply investigate the rejection mechanisms of xenotransplantation

6.2.1 Porcine carbohydrate antigens

NHPs often serve as transplant recipients to determine the efficacy of xenotransplantation. However, the expression profile of α-Gal in NHPs differs from that in humans (110). Therefore, data from NHPs

can only provide a reference for clinical xenotransplantation. Techniques have been developed to knock out multiple porcine genes (33, 111). However, recent data indicated that the loss of the non-Gal antigen, Neu5Gc, is associated with increased humoral rejection in pig-baboon kidney xenotransplants (112, 113). Therefore, an in-depth investigation of porcine carbohydrate antigens might provide a more comprehensive understanding of their roles in xenotransplantation.

6.2.2 The function and mechanism of novel molecules

In the most recent GTKO pig-baboon kidney xenotransplantation with an anti-CD40 mAb-based immunosuppressive regimen, results indicated that ATG and anti-CD20 mAb eliminated peripheral T and B lymphocytes and inhibited lymphocyte recovery; a decreased abundance of memory $CD8^+$ T cells might determine long-term outcomes (114). The hCD47 expression in porcine endothelial cells and podocytes reduced the phagocytic effects of human and baboon macrophages on porcine endothelial cells and podocytes by rectifying the inter-species incompatibility of CD47/Sirp-α signaling (115). Results suggest that the expression of human CD47 in donor pig renal glomerular cells might be an important strategy for preventing proteinuria after xenotransplantation. The results of an *in vivo* study suggested that porcine podocytes expressing hCD47 inhibit the development of albuminuria in GTKO/hCD47 Tg pig-baboon kidney xenotransplantation (116). The underlying mechanism deserves more intensive investigation.

6.2.3 Each type of immune cell involving xenograft rejection

In addition to T and B lymphocyte, monocyte, macrophages, neutrophils, and natural killer (NK) cells should all involve in the initiation and advancements of rejection and outcome of xenografts. Nevertheless, we are just scratching the surface of the iceberg about the function and mechanisms of each type of cells. For instance, NK cells may play an effector role by releasing cytotoxicity granules against xenogeneic cells, or an affector role on other immune cells through cytokine secretion (117), and much work need to be carried out to promote xenograft acceptance by driving NK cells (118).

6.2.4 The discrepancy in metabolism between kidney xenograft donors and human

Pigs, NHPs, and humans significantly differ biologically and physiologically (119–121). All findings suggested that specific immune tolerance induction or immunosuppression regimen need to be developed, and immune mechanism of chronic rejection needs to be explored from multi-angle exploration.

Accumulating evidence suggests that various metabolites and metabolic networks intersect with the induction, regulation, and maintenance of trained immunity (122). Metabolism and the immunological state are inextricably linked, and immunometabolism is recognized as a major mechanism that is central to adaptive and innate immune regulation (123). Now, whether, which, and how metabolites are involved in immune regulation of kidney xenografts remains to be determined. Kidney xenografts grow abnormally in hosts like any other xenograft. The threshold for the ratio of transplanted kidney volume to host body weight is 25 cm^2/kg; beyond this threshold, kidney xenografts become ischemic (124). This phenomenon reflects physiological differences between GTKO pigs and baboons and more importantly, a link between metabolism and the renal xenograft immune response. This is confirmed by the results that rituximab and CTLA4Ig might confer benefits in terms of symptomatic treatments (125–127).

7 Conclusion

Compared with the understanding of the alloimmune response, that of the heterologous immune mechanism is still in its infancy (128, 129). We believe that a deeper understanding of immunological theories and the development of techniques will continue to promote the progress of kidney xenotransplantation. Further studies of immunomechanisms in kidney xenotransplantation might help to promote the survival of kidney xenografts.

Author contributions

XH wrote the manuscript, and HX revised and reviewed the manuscript. All authors were involved in the creation of the manuscript and are responsible for the content of the work. All authors contributed to the article and approved the submitted version.

Funding

This study was funded by the National Natural Science Foundation of China (81272367).

Acknowledgments

We thank Editage (www.editage.cn) for English language editing.

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.

Publisher's note

References

1. Rodger D, Hurst D. Mathieu Jaboulay's (1860-1913) contribution to xenotransplantation. *Xenotransplantation* (2022) 29:e12765. doi: 10.1111/xen.12765

2. Montgomery RA, Stem JM, Lonze BE, Tatapudi VS, Mangiola M, Wu M, et al. Results of two cases of pig-to-human kidney xenotransplantation. *N Engl J Med* (2022) 386:1889–98. doi: 10.1056/NEJMoa2120238

3. Porrett PM, Bj O, Kumar V, Houp J, Anderson D, AC K, et al. First clinical-grade porcine kidney xenotransplant using a human decedent model. *Am J Transplant* (2022) 22:1037–53. doi: 10.1111/ajt.16930

4. Cooper DKC. Advancing xenotransplantation to the clinic: how relevant is the pig-to-nonhuman primate kidney transplantation model today? *Transplantation* (2022) 106:1717–9. doi: 10.1097/TP.0000000000004097

5. Sykes M, Sachs DH. Progress in xenotransplantation: overcoming immune barriers. *Nat Rev nephrol* (2022) 18:745–61. doi: 10.1038/s41581-022-00624-6

6. Anderson DJ, Locke JE. Progress towards solving the donor organ shortage. *Nat Rews Neph* (2023) 19:83–4. doi: 10.1038/s41581-022-00664-y

7. Rowlands DTJr., Kirkpatrick CH, Vatter AE, Wilson WE. Immunologic studies in human organ transplantation. IV. Serologic and pathologic studies following heterotransplantation of the kidney. *Am J Pathol* (1967) 50:605–22.

8. Owen ER. Prolonged survival in heterografted kidneys with transplantation antigen pretreatment. *Nature* (1968) 219:970–1.

9. Merkel FK, Bier M, Beavers CD, Merriman WG, Wilson C, Starzl TE. Modification of xenograft response by selective plasmapheresis. *Transplant Proc* (1971) 3:534–7.

10. Moberg AW, Shons AR, Gewurz H, Mozes M, Najarian JS. Prolongation of renal xenografts by the simultaneous sequestration of preformed antibody, inhibition of complement, coagulation and antibody synthesis. *Transplant Proc* (1971) 3:538–41.

11. Wood C, Kabat EA, Murphy LA, Goldstein IJ. Immunochimical studies of the combining sites of the two isolectins, A4 and B4, isolated from Bandeiraea simplicifolia. *Arch Biochem Biophys* (1979) 198:1–11. doi: 10.1016/0003-9861(79)90389-8

12. Good AH, Cooper DK, Malcolm AJ, Ippolito RM, Koren E, Neethling FA, et al. Identification of carbohydrate structures that bind human antiporcine antibodies: implications for discordant xenografting in humans. *Transplant Proc* (1992) 24:559–62.

13. Cooper DK, Good AH, Koren E, Orial R, Malcolm AJ, Ippolito RM, et al. Identification of alpha-galactosyl and other carbohydrate epitopes that are bound by human anti-pig antibodies: relevance to discordant xenografting in man. *Transpl Immunol* (1993) 1:198–205. doi: 10.1016/0966-3274(93)90047-C

14. Oriol R, Ye Y, Koren E, Cooper DK. Carbohydrate antigens of pig tissues reacting with human natural antiboies as potential targets for hyperacute vascular rejection in pig-to-man organ xenotransplantation. *Transplantation* (1993) 56:1433–42. doi: 10.1097/00007890-199312000-00031

15. Galili U. Interaction of the natural anti-Gal antibody with alpha-galactosyl epitopes: a major obstacle for xenotransplantation in humans. *Immunol Today* (1993) 14:480–2. doi: 10.1016/0617-5699(93)90261-i

16. Sandrin MS, Vaughan HA, Dabkowski PL.McKenzie IF. Anti-pig IgM antibodies in human serum react predominantly with Gal (alpha 1-3) Gal epitopes. *Proc Natl Acad Sci USA* (1993) 90:11391–5. doi: 10.1073/pnas.90.23.11391

17. McKenzie IF, Xing PX, Vaughan HA, Prenzoska J, Dabkowski PL, Sandrin MS. Distribution of the major xenoantigen (gal (alpha 1-3) gal) for pig to human xenografts. *Transpl Immunol* (1994) 2:81–6. doi: 10.1016/0966-3274(94)90032-9

18. Vaughan HA, Loveland BE, Sandrin MS. GALα(1,3)GAL is the major xenoepitope expressed on pig endothelial cells recognized by naturally occurring cytotoxic human antibodies. *Transplantation* (1994) 58:879–82. doi: 10.1097/000078-199410270-00003

19. Cairns T, Lee J, Goldberg L, Cook T, Simpson P, Sparckman D, et al. Inhibition of the pig to human xenograft reaction, using soluble Gal alpha 1-3Gal and Gal alpha 1-3Gal beta 1-4GleNAc. *Transplantation* (1995) 60:1202–7. doi: 10.1097/00007890-199512150-00004

20. Nitta K. Ex vivo spleen and kidney absorption of xenoreactive natural antibodies decreases severity of hyperacute rejection in pig-to-dog renal xenotransplantation. *Hiroshima J Med Sci* (1996) 45:119–25.

21. Gollackner B, Knosalla C, Houser S, Mauiyyed S, Buhler L, Kawai T, et al. Pig kidney transplantation in baboons treated intravenously with a bovine serum albumin-Galalpha1-3Gal conjugate. *Xenotransplantation* (2003) 10:606–14. doi: 10.1034/j.1399-3089.2003.00065.x

22. Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, et al. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science* (2002) 295:1089–92. doi: 10.1126/science.1068228

23. Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, et al. Production of alpha 1,3-galactosyltransferase-deficient pigs. *Science* (2003) 299:411–4. doi: 10.1126/science.1078942

24. Kolber-Simonds D, Lai L, Watt SR, Denaro M, Arn S, Augenstein ML, et al. Production of alpha-1,3-galactosyltransferase null pigs by means of nuclear transfer with fibroblasts bearing loss of heterozygosity mutations. *Proc Natl Acad Sci USA* (2004) 101:7335–40. doi: 10.1073/pnas.0307819101

25. Chen G, Qian H, Starzl T, Sun H, Garcia B, Wang X, et al. Acute rejection is associated with antibodies to non-Gal antigens in baboon using Gal-knockout pig kidneys. *Nat Med* (2005) 11:1295–8. doi: 10.1038/nm1330

26. Kirkeby S, Mikkelsen HB. Distribution of the alphaGal- and non-alphaGal T-antigens in the pig kidney: potential targets for rejection in pig-to-man xenotransplantation. *Immunol Cell Biol* (2008) 86:363–71. doi: 10.1038/icb.2008.1

27. Miwa Y, Kobayashi T, Nagasaka T, Liu D, yu M, Yokoyama I, et al. Are N-glycolylneuraminic acid (Hanganutziu-Deicher) antigens important in pig-to-human xenotransplantation? *Xenotransplantation* (2004) 11:247–53. doi: 10.1111/j.1399-3089.2004.00126.x

28. Martin MJ, Rayner JC, Gagneux P, Barnwell JB, Varki A. Evolution of human-chimpanzee differences in malaria susceptibility: relationship to human genetic loss of N-glycolylneuraminic acid. *Proc Natl Acad Sci USA* (2005) 102:12819–24. doi: 10.1073/pnas.0503819102

29. Burlak C, Paris LL, Lutz AJ, Sider RA, Estrada J, Li P, et al. Reduced binding of human antibodies to cells from GGTA1/CMAH KO pigs. *Am J Transplant* (2014) 14:1895–900. doi: 10.1111/ajt.12744

30. Byme GW, Du Z, Stalboerger P, Kogelberg H, McGregor CGA. Cloning and expression of porcine β1,4 N-acetylgalactosaminyl transferase encoding a new xenoreaction antigen. *Xenotransplantation* (2014) 21:543–54. doi: 10.1111/xen.12124

31. Byrne G, Ahmad-Villiers S, Du Z, McGregor C, et al. B4GALNT2 and xenotransplantation: a newly appreciated xenogeneic antigen. *Xenotransplantation* (2018) 25(5):e12394. doi: 10.1111/xen.12394

32. Sato M, Miyoshi K, Nagao Y, Nishi Y, Ohtsuka M, Nakamura S, et al. The combinational use of CRISPR/Cas9-based gene editing and targeted toxin technology enables efficient biallelic knockout of the α-1,3-galactosyltransferase gene in porcine embryonic fibroblasts. *Xenotransplantation* (2014) 21:291–300. doi: 10.1111/xen.12089

33. Martens GR, Teyes LM, Li P, Butler JR, Ladowski JM, Estrada JL, et al. Humoral reactivity of renal transplant- Waitlisted patients to cells from GGTA1/CMAH/B4GalNT2, and SLA class I knockout pigs. *Transplantation* (2017) 101:e86–92. doi: 10.1097/TP.0000000000001646

34. Fischer K, Rieblinger B, Hein R, Sfriso R, Zuber J, Fischer A, et al. Viable pigs after simultaneous inactivation of porcine MHC class I and three xenoreactive antigen genes GGTA1, CMAH and B4GALTN2. *Xenotransplantation* (2020) 27:e12560. doi: 10.1111/xen.12560

35. Feng H, Li T, Du J, Xia Q, Wang L, Chen S, et al. Both natural and induced anti-Sda antibodies play important roles in GTKO pig-to-rhesus monkey xenotransplantation. *Front Immunol* (2022) 13:849711. doi: 10.3389/fimmu.2022.849711

36. Bello-Gil D, Olivera-Ardid S, Tuzikov AB, Costa C, Bovin NV, Mañezl. Antibodies against hyaluronan oligosaccharides in xenotransplantation. *Xenotransplantation* (2023) 30:e12799. doi: 10.1111/xen.12799

37. Martens GR, Reyes L, Li P, Butler JR, Ladowski JM, Estrada JL, et al. Humoral reactivity of renal transplant-waitlisted patients to cells from GGTA1/CMAH/B4GalNT2, and SLA class I knockout pigs. *Transplantation* (2017) 101:e86–e92. doi: 10.1097/TP.0000000000001646

38. Ladowski JM, Reyes L, Martens GR, Butler JR, Wang Z, Eckhoff DE, et al. Swine leukocyte antigen class II is a xenoantigen. *Transplantation* (2018) 102:249–54. doi: 10.1097/TP.0000000000001924

39. Ladowski JM, Martens GR, Reyes LM, Wang Z, Eckhoff DE, Hauptfeld-Dolejsek V, et al. Examining the biosynthesis and xenoantigenicity of class II swine leukocyte antigen proteins. *J Immunol* (2018) 200:2957–64. doi: 10.4049/jimmunol.1800022

40. Hein R, Sake HJ, Pokoyski C, Hundrieser J, Brinkmann A, Baars W, et al. Triple (GGTA1, CMAH, B2M) modified pigs expressing ans SLA class I low phenotype-effects on immune status and susceptibility to human immune responses. *Am J Transplant* (2020) 20:988–98. doi: 10.1111/ajt.15710

41. Li W, Chen P, Zhao Y, Cao M, Hu W, Pan L, et al. Human IL-17 and TNF-α additively or synergistically regulate the expression of proinflammatory genes, coagulation-related genes, and tight junction genes in porcine aortic endothelial cells. *Front Immunol* (2022) 30:857311. doi: 10.3389/fimmu.2022.857311

42. Schmalkuche K, Schwinzer R, Wenzel N, Valdivia E, Petersen B, Blasczyk R, et al. Downregulation of swine leukocyte antigen expression decreases the strength of xenogeneic immune responses towards renal proximal tubular epithelial cells. *Int J Mol Sci* (2023) 24:12711. doi: 10.3390/ijms241612711

43. Clark EA. CD40: a cytokine receptor in search of a ligand. *Tissue Antigens* (1990) 36:33–6. doi: 10.1111/j.1399-0039.1990.tb01795.x

44. Armitage RJ, Fanslow WC, Strockbine L, Sato TA, Clifford KN, Macduff BM, et al. Molecular and biological characterization of a murine ligand for CD40. *Nature* (1992) 357:80–2. doi: 10.1038/357080a0

45. Noelle RJ, Roy M, Shepherd DM, Stamenkovic I, Ledbetter JA, Aruffo A. A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. *Proc Natl Acad Sci USA* (1992) 89:6550–4. doi: 10.1073/pnas.89.14.6550

46. Hollenbaugh D, Grosmaire LS, Kullas CD, Chalupny NJ, Braesch-Andersen S, Noelle RJ, et al. The human T cell antigen gp39, a member of the TNF gene family, is a

ligand for the CD40 receptor: expression of a soluble form of gp39 with B cell costimulatory activity. *EMBO J* (1992) 11:4313–21. doi: 10.1002/j.1460-2075.1992.tb05330.x

47. Spriggs MK, Armitage RJ, Strockbine L, Clifford KN, Macduff BM, Sato TA, et al. Recombinant human CD40 ligand stimulates B cell proliferation and immunoglobulin E secretion. *J Exp Med* (1992) 176:1543–50. doi: 10.1084/jem.176.6.1543

48. Nonoyama S, Hollenbaugh D, Aruffo A, Ledbetter JA, Ochs HD. B cell activation *via* CD40 is required for specific antibody production by antigen-stimulated human B cells. *J Exp Med* (1993) 178:1097–102. doi: 10.1084/jem.178.3.1097

49. Tsubata T, Wu J, Honjo T. B-cell apoptosis induced by antigen receptor crosslinking is blocked by a T-cell signal through CD40. *Nature* (1993) 364:645–8. doi: 10.1038/364645a0

50. Van den Eertwegh AJ, Noelle RJ, Roy M, Shepherd DM, Aruffo A, Ledbetter JA, et al. *In vivo* CD40-gp39 interactions are essential for thymus-dependent humoral immunity. I *In vivo* expression of CD40 ligand, cytokines, and antibody production delineates sites of cognate T-B cell interactions. *J Exp Med* (1993) 178:1555–65. doi: 10.1084/jem.178.5.1555

51. Foy TM, Shepherd DM, Durie FH, Aruffo A, Ledbetter JA, Noelle RJ. *In vivo* CD40-gp39 interactions are essential for thymus-dependent humoral immunity. II. Prolonged suppression of the humoral immune response by an antibody to the ligand for CD40, gp39. *J Exp Med* (1993) 178:1567–75. doi: 10.1084/jem.178.5.1567

52. Foy TM, Page DM, Waldschmidt TJ, Schoneveld A, Masters SR, Tygrett L, et al. An essential role for gp39, the ligand for CD40, in thymic selection. *J Exp Med* (1995) 182:1377–88. doi: 10.1084/jem.182.5.1377

53. Pearson TC, Trambley J, Odom K, Anderson DC, Cowan S, Bray R, et al. Anti-CD40 therapy extends renal allograft survival in rhesus macaques. *Transplantation* (2002) 74:933–40. doi: 10.1097/00007890-200210150-00006

54. Watanabe M, Kumagai-Braesch M, Yao M, Thunberg S, Berglund D, Sellberg F, et al. Ex vivo generation of donor antigen-specific immunomodulatory cells: a comparison study of anti-CD80/86 mAbs and CTLA4-Ig costimulatory blockade. *Cell Transplant* (2018) 27:1692–704. doi: 10.1177/0963689718794642

55. Lee RS, Yamada K, Womer KL, Pillsbury EP, Allison KS, Marolewski AE, et al. Blockade of CD28-B7, but not CD40-CD154, prevents costimulation of allogeneic porcine and xenogeneic human anti-porcine T cell responses. *J Immunol* (2000) 164:3434–44. doi: 10.4049/jimmunol.164.6.3434

56. BÜhler L, Yamada K, Kitamura H, Alwayn IP, Basker M, Appel 3JZ, et al. Pig kidney transplantation in baboons: anti-Gal(alphα)1-3Gal IgM alone is associated with acute humoral xenograft rejection and disseminated intravascular coagulation. *Transplantation* (2001) 72:1743–52. doi: 10.1097/00007890-200112150-00007

57. Knosalla C, Ryan D, Moran K, Gollackner B, Schuler W, DH S, et al. Initial experience with the human anti-human CD154 monoclonal antibodt, ABI793, in pig-to-baboon xenotransplantation. *Xenotransplantation* (2004) 11:353–60. doi: 10.1111/j.1399-3089.2004.00148.x

58. Bottino R, Knoll MF, Graeme-Wilson J, Klein EC, Ayares D, Trucco M, et al. Safe use of anti-CD154 monoclonal antibody in pig islet xenotransplantation in monkeys. *Xenotransplantation* (2017) 24:10. doi: 10.1111/xen.12283

59. Wu G, Pfeiffer S, Schröder C, Zhang T, Nguyen BN, Lea W, et al. Co-stimulation blockade targeting CD154 and CD28/B7 modulates the induced antibody response after a pig-to-baboon cardiac xenograft. *Xenotransplantation* (2005) 12:197–208. doi: 10.1111/j.1399-3089.2005.00221.x

60. BÜhler I, Alwayn IP, Basker M, Oravec G, Thall A, White-Scharf ME, et al. CD40-CD154 pathway blockade requires host macrophages to induce homural unresponsiveness to pig hematopoietic cells in baboons. *Transplantation* (2001) 72:1759–68. doi: 10.1097/00007890-200112150-00009

61. Thopmson P, Cardona K, Russell M, Badell IR, Shaffer V, Korbutt G, et al. CD40-specific costimulation blockade enhances neonatal porcine islet survival in nonhuman primates. *Am J Transplant* (2011) 11:947–57. doi: 10.1111/j.1600-6143.2011.03509.x

62. Thompson P, Badell IR, Lowe M, Turner A, Cano J, Avila J, et al. Alterative immunomodulatory strategies for xenotransplantation: CD40/154 pathway-sparing regimens promote xenograft survival. *Am J Transplant* (2012) 12:1765–75. doi: 10.1111/j.1600-6143.2012.04031.x

63. Cardona K, Korbutt GS, Milas Z, Lyon J, Cano J, Jiang W, et al. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat Med* (2006) 12:304–6. doi: 10.1038/nm1375

64. Mohiuddin MM, Singh AK, Corcoran PC, Iii MLT, Clark T, Lweis BG, et al. Chimeric 2C10R4 anti-CD40 antibody therapy is critical for long-term survival of GTKO.hCD46.h TBM pig-to-primate cardiac xenograft. *Nat Commun* (2016) 7:11138. doi: 10.1038/ncomms11138

65. Shin JS, Kim JM, Kim JS, Min BH, Kim YH, Kim HJ, et al. Long-term controls of diabetes in immunosuppressed nonhuman primates (NHP) by the transplantation of adult porcine islets. *Am J Tranpsplant* (2015) 15:2837–50. doi: 10.1111/ajt.13345

66. Yin D, Ma L, Shen J, Byrne GW, Logan JS, Chong ASF. CTLA-4Ig in combination with anti-CD40L prolongs xenograft survival and inhibits anti-gal ab production in GT-Ko mice. *Am J Transplant* (2002) 2:41–7. doi: 10.1034/j.1600-6143.2002.020108.x

67. Cooper DKC, Foote JB, Javed M, Nguyen HQ, Bikhet MH, Hansen-Estruch C, et al. Initial evidence that blockade of the CD40/CD154 costimulation pathway alone is sufficient as maintenance therapy in xenotransplantation. *Xenotransplantation* (2021) 28:e12721. doi: 10.1111/xen.12721

68. Kennedy SP, Rollins SA, Burton WV, Sims PJ, Bothwell AL, Squinto SP, et al. Protection of porcine aortic endothelial cells from complement-mediated cell lysis and activation by recombinant human CD59. *Transplantation* (1994) 57:1494–501. doi: 10.1097/00007890-199405270-00017

69. Kroshus TJ, Bolman 3RM, Dalmasso AP, Rollins SA, Guilmette ER, Williams BL, et al. Expression of human CD59 in transgenic pig organs enhances organ survival in an ex vivo xenogeneic perfusion model. *Transplantation* (1996) 61:1513–21. doi: 10.1097/00007890-199605270-00018

70. Neimann H, Rath D. Progress in reproductive biotechnology in swine. *Theriogenology* (2001) 56:1291–304. doi: 10.1016/s0093-691x(01)00630-6

71. Baldan N, Rigotti P, Calabrese F, Cadrobbi R, Dedja A, Iacooetti I, et al. Ureteral stenosis in HDAF pig-to primate renal xenotransplantation: a phenomenon related to immunological events? *Am J Transplant* (2004) 4:475–81. doi: 10.1111/j.1600-6143.2004.00407.x

72. Higginobotham L, Mathews D, Breeden CA, Song M, Farris 3AB, Larsen CP, et al. Pre-transplant antibody screening and anti-CD154 costimulation blockade promote long-term xenograft survival in a pig-to-primate kidney transplant model. *Xenotransplantation* (2015) 22:221–30. doi: 10.1111/xen.12166

73. Kim H, Hawthone WJ, Kang HJ, Lee YJ, Hwang J, Hurh S, et al. Human thrombomodulin regulates complement activation as well as the coagulation cascade in xeno-immune response. *Xenotransplantation* (2015) 22:260–72. doi: 10.1111/xen.12173

74. Hara H, Iwase H, Nguyen H, Miyagawa Y, Kuravi K, Foote JB, et al. Stable expression of the human thrombomodulin transgene in pig endothelial cells is associated with a reduction in the inflammatory response. *Cytokine* (2021) 148:155580. doi: 10.1016/j.cyto.2021.155580

75. Robson S, Wu Y, Sun X, Knosalla C, Dwyer K, Enjyoji K. Extonucleotidases of CD39 family modulate vascular inflammation and thrombosis in transplantation. *Semin Thromb Hemost* (2005) 31:217–33. doi: 10.1055/s-2005-869527

76. Iwase H, Liu H, Wijkstrom M, Zhou H, Singh J, Hara H, et al. Pig kidney graft survival in a baboon for 136 days: longest life-supporting organ graft survival to date. *Xenotransplantation* (2015) 22:302–9. doi: 10.1111/xen.12174

77. Kim SC, Mathews DV, Breeden CP, Higginbotham LB, Ladowski J, Martens G, et al. Long-term survival of pig-to-rhesus macaque renal xenogrfts is dependent on CD4 T cell depletion. *Am J Transplant* (2019) 19:2174–85. doi: 10.1111/ajt.15329

78. Lee LA, Gritsch HA, Sergio JJ, Arn JS, Glaser RM, Sablinski T, et al. Specific tolerance across a discordant xenogeneic transplantation barrier. *Proc Natl Acad Sci USA* (1994) 91:10864–7. doi: 10.1073/pnas.91.23.10864

79. Zhao Y, Sergio JJ, Swenson K, Arn JS, Sachs DH, Sykes M. Positive and negative selection of functional mouse CD4 cells by porcine MHC in pig thymus grafts. *J Immunol* (1997) 159:2100–7. doi: 10.4049/jimmunol.159.5.2100

80. Zhao Y, Fishman JA, Sergio JJ, Oliveros JL, Pearson DA, Szot GL, et al. Immune restoration by fetal pig thymus grafts in T cell-depleted, thymectomized mice. *J Immunol* (1997) 158:1641–9. doi: 10.4049/jimmunol.158.4.1641

81. Zhao Y, Swenson K, Sergio JJ, Sykes M. Pig MHC mediated positive selection of mouse CD4+ T cells with a mouse MHC-restricted TCR in pig thymus grafts. *J Immunol* (1998) 161:1320–6. doi: 10.4049/jimmunol.161.3.1320

82. Yamada K, Gianello PR, Ierino FL, Lorf T, Shimizu A, Meehan S, et al. Role of the thymus in transplantation tolerance in miniature swine. I. Requirement of the thymus for rapid and stable induction of tolerance to class I-mismatched renal allografts. *J Exp Med* (1997) 186:497–506. doi: 10.1084/jem.186.4.497

83. Yamada K, Ierino FL, Gianello PR, Shimizu A, Colvin RB, Sachs DH. Role of the thymus in transplantation tolerance in miniature swine. III. Surgical manipulation of the thymus interferes with stable induction of tolerance to class I-mismatched renal allografts. *Transplantation* (1999) 67:1112–9. doi: 10.1097/00007890-199904270-00005

84. Yamada K, Shimizu A, Inerino FL, Utsugi R, Barth RN, Esnaola N, et al. Thymic transplantation in miniature swine. I. Development and function of the "thymokidney". *Transplantation* (1999) 68:1684–92. doi: 10.1097/00007890-199912150-00011

85. Yamada K, Gianello PR, Ierino FL, Fishbein J, Lorf T, Shimizu A, et al. Role of the thymus in transplantation tolerance in miniature swine. II. Effect of steroids and age on the induction of tolerance to class I mismatched renal allografts. *Transplantation* (1999) 67:458–67. doi: 10.1097/00007890-199902150-00020

86. Yamada K, Shimizu A, Utsugi R, Ierino FL, Gargollo P, Haller GW, et al. Thymic transplantation in miniature swine. II. Induction of tolerance by transplantation of composite thymokidneys to thymectomized recipients. *J Immunol* (2000) 164:3079–86. doi: 10.4049/jimmunol.164.6.3079

87. Barth RN, Yamamoto S, LaMattina JC, Kumagai N, Kitamura H, Vagefi PA, et al. Xenogeneic thymokidney and thymic tissue transplantation in a pig-to-baboon model: I. evidence for pig-specific T-cell unresponsiveness. *Transplantation* (2003) 75:1615–24. doi: 10.1097/01.TP.0000064335.50622.20

88. Yamada K, Yazawa K, Shimizu A, Iwanaga T, Hisashi Y, Nuhn M, et al. Marked prolongation of porcine renal xenograft survival in baboons through the use of alpha1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. *Nat Med* (2005) 11:32–4. doi: 10.1038/nm1172

89. Griesemer AD, Hirakata A, Shimizu A, Moran S, Tena A, Iwaki H, et al. Results of gal-knockout porcine thymokidney xenografts. *Am J Transplant* (2009) 9:2669–78. doi: 10.1111/j.1600-6143.2009.02849.x

90. Fudaba Y, Onoe T, Chittenden M, Shimizu A, Shaffer JM, Bronson R, et al. Abnormal regulatory and effector T cell function predispose to autoimmunity following

xenogeneic thymic transplantation. *J Immunol* (2008) 181:7649–59. doi: 10.4049/jimmunol.181.11.7649

91. Sekijima M, Sahara H, Shimizu A, Iwanaga T, Murokawa T, Ariyoshi Y, et al. Preparation of hybrid porcine thymus containing non-human primate thymic epithelial cells in minature swine. *Xenotransplantation* (2019) 26:e12543. doi: 10.1111/xen.12543

92. Llore Np, Bruestle KA, Griesemer A. Xenotransplantation tolerance: applications for recent advances in modified swine. *Curr Opin Organ Transplant* (2018) 23:642–8. doi: 10.1097/MOT.0000000000000585

93. Zhao Y, Rodriguez-Barbosa JI, Zhao G, Shaffer J, Arn JS, Sykes M. Mutaration and function of mouse T-cells with a transgenic TCR positively selected by highly disparate xenogeneic porcine MHC. *Cell Mol Biol (Noisy-le-grand).* (2001) 47:217–28.

94. Nikolic B, Gardner JP, Scadden DT, Arn JS, Sachs DH, Sykes M. Normal development in porcine thymus grafts and specific tolerance of human T cells to porcine donor MHC. *J Immunol* (1999) 162:3402–7. doi: 10.4049/jimmunol.162.6.3402

95. Zhao Y, Rodriguez-Barbosa JI, Shimizu A, Sachsss DH, Sykes M. Despite efficient intrathymic negative selection of host-reactive T cells, autoimmune disease may develop in porcine thymus-grafted athymic mice: evidence for failure of regulatory mechanisms suppressing autoimmunity. *Transplantation* (2003) 75:1832–40. doi: 10.1097/01.TP.0000065292.20062.F0

96. Fudaba Y, Onoe T, Chittenden M, Shimizu A, Shaffer JM, Bronson R, et al. Abnormal regulatory and effector T cell function predispose to autoimmunity following xenogeneic thymic transplantation. *J Immunol* (2008) 181:7649–59. doi: 10.4049/jimmunol.181.11.7649

97. Nauman G, Borsotti C, Danzl N, Khosravi-Maharlooei M, Li H, Chavez E, et al. Reduced positive selection of a human TCR in a swine thymus using a humanized mouse model for xenotolerance induction. *Xenotransplantation* (2020) 27:e12558. doi: 10.1111/xen.12558

98. Kawai T, Sachs DH. Tolerance induction: hematopoietic chimerism. *Curr Opin Organ Transplant* (2013) 18:402–7. doi: 10.1097/MOT.0b013e328363621d

99. Duran-Struck R, Sondermeijer HP, Bühler L, Alomso-Guallart P, Zitsman J, Kato Y, et al. Effect of ex vivo-expanded recipient regulatory T cells on hematopoietic chimerism and kidney allograft tolerance across MHC barriers in cynomolgus macaques. *Transplantation* (2017) 101:274–83. doi: 10.1097/TP.0000000000001559

100. Kawai T, Cosimi AB, Spitzer TR, Tolkoff-Rubin N, Suthanthiran M, Saidman SL, et al. HLA-mismatched renal transplantaiton without maintenance immunosuppression. *N Engl J Med* (2008) 358:353–61. doi: 10.1056/NEJMoa071074

101. LoCascio SA, Morokata T, Chittenden M, Preffer FI, Dombkowski DM, Andreola G, et al. Mixed chimerism, lymphocyte recovery, and evidence for early donor-specific unresponsiveness in patients receiving combined kidney and bone marrow transplantation to induce tolerance. *Transplantation* (2010) 90:1607–15. doi: 10.1097/TP.0b013e3181ffbaff

102. Andreola G, Chittenden M, Shaffer J, Cosimi AB, Kawai T, Cotter P, et al. Mechanisms of donor-specific tolerance in recipients of haplodentical combined bone marrow/kidney transplantation. *Am J Transplant* (2011) 11:1236–47. doi: 10.1111/j.1600-6143.2011.03566.x

103. Simon AR, Schröder C, Martin U, Tessmann R, Templin C, Laaf G, et al. Induction of long-term peripheral microchimerism in non-human primates in a model of xenogeneic peripheral tolerance induction. *Ann Transplant* (2002) 7:40–5.

104. Griesemer A, Liang F, Hirakata A, Hirsh E, Lo D, Okumi M, et al. Occurrence of specific humoral non-responsiveness to swine antigens following administration of GalT-Ko bone marrow to baboons. *Xenotransplantation* (2010) 17:300–12. doi: 10.1111/j.1399-3089.2010.00600.x

105. Tasaki M, Wamala I, Tena A, Villani V, Sekijima M, Pathiraja V, et al. High incidence of xenogenic bone marrow engraftment in pig-to baboon intra-bone bone marrow transplantation. *Am J Transplant* (2015) 15:974–83. doi: 10.1111/ajt.13070

106. Tena AA, Sachs DH, Mallard C, Yang Y, Tasaki M, Farkash E, et al. Prolonged survival of pig skin on baboons after administration of pig cells expressing human CD47. *Transplantation* (2017) 101:316–21. doi: 10.1097/TP.0000000000001267

107. Denner J. Virus safety of xenotransplantation. *Viruses* (2022) 14:1926. doi: 10.3390/v14091926

108. Kuscu C, Kuscu C, Bajwa A, Eason JD, Maluf D, Mas VR. Applications of CRISPR technologies in transplantation. *Am J Transplant* (2020) 20:3285–93. doi: 10.1111/ajt.16095

109. Deng J, Yang L, Wang Z, ouyang H, Yu H, Yuan H, et al. Advance of genetically modified pigs in xeno-transplantation. *Front Cell Dev Biol* (2022) 10:1033197. doi: 10.3389/fcell.2022.1033197

110. Mckenzie IF, Xing PX, Vaughan HA, Prenzoska J, Dabkowski PL, Sandrin MS. Distribution of the major xenoantigen (gal (alpha 1-3)gal) for pig to human xenografts. *Transpl Immunol* (1994) 2:81–6. doi: 10.1016/0966-3274(94)90032-9

111. Yoon S, Lee S, Park C, Choi H, Yoo M, Lee SC, et al. An efficacious transgenic strategy for triple knockout of xeno-reactive antigen genes GGTA1, CMAH, and B4GALNT2 from Jeju Native Pigs. *Vaccines (Basel)* (2022) 10:1503. doi: 10.3390/vaccines10091503

112. Iwase H, Jagdale A, Yamamoto T, Bikhet MH, Nguyen H, Ezzelarab M, et al. Evidencee suggesting that deletion of expression of N-glycolylneuraminic acid (Neu5Gc) in the organ-source pig is associated with increased antibody-mediated rejection of kidney transplants in baboons. *Xenotransplantation* (2021) 28:e12700. doi: 10.1111/xen.12700

113. Foote JB, Jagdale A, Yamamoto T, Hara H, Bikhet M, Schuurman HJ, et al. Histopathology of pig kidney grafts with/without expression of the carbohydrate Neu5Gc in immunosuppressed baboons. *Xenotransplantation* (2021) 28:e12715. doi: 10.1111/xen.12715

114. Jagdale A, Nguyen H, Iwase H, Foote JB, Yamamoto T, Javed M, et al. T and B lymphocyte dynamics after genetically-modified pig-to-baboon kidney xenotransplantation with an anti-CD40mAb-based immunosuppressive regimen. *Transpl Immunol* (2022) 71:101545. doi: 10.1016/j.trim.2022.101545

115. Nomura S, Ariyoshi Y, Watanabe H, Pomposelli T, Takeuchi K, Garcia G, et al. Transgenic expression of human CD47 reduces phagocytosis of porcine endothelial cells and podocytes by baboon and human macrophages. *Xenotransplantation* (2020) 27:e12549. doi: 10.1111/xen.12549

116. Takeuchi K, Ariyoshi Y, Shimizu A, Okumura Y, Cara-Fuentes G, Garcia GE, et al. Expression of human CD47 in pig glomeruli prevents proteinuria and prolongs graft survival following pig-to-baboon xenotransplantation. *Xenotransplantation* (2021) 28:e12708. doi: 10.1111/xen.12708

117. Puga Yung G, Schneider MKJ, Seebach JD. The role of NK cells in pig-to-human xenotransplantation. *J Immunol Res* (2017) 2017:4627384. doi: 10.1155/2017/4627384

118. Lopez KJ, Cross-Najafi AA, Farag K, Obando B, Thadasina D, Isidan A, et al. Strategies to induce natural killer cell tolerance in xenotransplantation. *Front Immunol* (2022) 13:941880. doi: 10.3389/fimmu.2022.941880

119. Tatapudi VS, Griesemer AD. Physiologic considerations of pig-to-human kidney xenotransplantation. *Curr Opin Nephrol Hypertens* (2023) 32:193–8. doi: 10.1097/MNH.0000000000000858

120. Iwase H, Klein EC, Cooper DKC. Physiologic aspects of pig kidney transplantation in nonhuman primates. *Comp Med* (2018) 68:332–40. doi: 10.30802/AALAS-CM-17-000117

121. Cohen AJ, Larson TS, Dean P, Logan J, Diamond L, McGregor CG, et al. Renal Physiol pig-to-baboon xenografts. *Transplant Proc* (2001) 33:727–8. doi: 10.1016/s0041-1345(00)02226-0

122. Fanucchi S, Dominguez-Andrés J, Joosten LAB, Netea MG. The intersection of epigenetics and metabolism in trained immunity. *Immunity* (2021) 54:32–43. doi: 10.1016/j.immuni.2020.10.011

123. Makowski L, Chaib M, Rathmell JC. Immunometabolism: from basic mechanisms to translation. *Immunol Rev* (2020) 295:5–14. doi: 10.1111/imr.12858

124. Tanabe T, Watanabe H, Shan JA, Sahara H, Shimizu A, Nomura S, et al. Role of intrinsic (graft) versus extrinsic (host) factors in the growth of transplanted organs following allogeneic and xenogeneic transplantation. *Am J Transplant* (2017) 17:1778–90. doi: 10.1111/ajt.14210

125. Yamada K, Shah JA, Tanabe T, Lanaspa MA, Johnson RJ. Xenotransplantation: where are we with potential kidney recipients? Recent progress and potential future clinical trials. *Curr Transplant Rep* (2017) 4:101–9. doi: 10.1007/s40472-017-0149-6

126. Bikhet M, Iwase H, Yamamoto T, Jagdale A, Foote JB, Ezzelarab M, et al. What therapeutic regimen will be optimal for initial clinical trials of pig organ transplantation? *Transplantation* (2021) 1054:1143–55. doi: 10.1097/TP.0000000000003622

127. Tasaki M, Shimizu A, Hanekamp I, Torabi R, Villani V. Rituximab treatment prevents the early development of proteinuria following pig-to-baboon xeno-kidney transplantation. *J Am Soc Nephrol.* (2014) 25:737–44. doi: 10.1681/ASN.2013040363

128. Maeda A, Kogata S, Toyama C, Lo P, Okamatsu C, Yamamoto R, et al. The innate cellular immune response in xeneotransplantation. *Front Immunol* (2022) 13:858604. doi: 10.3389/fimmu.2022.858604

129. Li T, Jiang H, Liu H, Cooper DKC, Wang Y. Extracellular histones and xenotransplantation. *Xenotransplantation* (2020) 27:e12618. doi: 10.1111/xen.12618

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 30 January 2024
DOI 10.3389/fimmu.2024.1279050

OPEN ACCESS

EDITED BY
Burcin Ekser,
Indiana University Bloomington, United States

REVIEWED BY
Ping Li,
Indiana University School of medicine, United States
Lisha Mou,
Shenzhen Second People's Hospital, China

*CORRESPONDENCE
Constanca Figueiredo
Figueiredo.Constanca@mh-hannover.de

RECEIVED 17 August 2023
ACCEPTED 11 January 2024
PUBLISHED 30 January 2024

CITATION
Schmalkuche K, Rother T, Besli S, Schwinzer R, Blasczyk R, Petersen B and Figueiredo C (2024) Human PD-L1 overexpression decreases xenogeneic human T-cell immune responses towards porcine kidneys.
Front. Immunol. 15:1279050.
doi: 10.3389/fimmu.2024.1279050

Human PD-L1 overexpression decreases xenogeneic human T-cell immune responses towards porcine kidneys

Katharina Schmalkuche[1,2], Tamina Rother[1], Sevval Besli[1], Reinhard Schwinzer[2,3], Rainer Blasczyk[1], Björn Petersen[2,4] and Constanca Figueiredo[1,2*]

[1]Institute of Transfusion Medicine and Transplant Engineering, Hannover Medical School, Hannover, Germany, [2]Transregional Collaborative Research Centre 127, Hannover Medical School, Hannover, Germany, [3]Transplantation Laboratory, Clinic for General, Visceral and Transplantation-Surgery, Hannover Medical School, Hannover, Germany, [4]Department of Biotechnology, Institute of Farm Animal Genetics, Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Neustadt am Rübenberge, Germany

Xenotransplantation offers a promising alternative to circumvent the lack of donated human organs available for transplantation. Different attempts to improve the survival of xenografts led to the generation of transgenic pigs expressing various combinations of human protective genes or knocked out for specific antigens. Currently, testing the efficiency of porcine organs carrying different genetic modifications in preventing xenogeneic immune responses completely relies on *in vitro* assays, humanized mouse models, or non-human primate transplantation models. However, these tests are often associated with major concerns due to reproducibility and generation of insufficient data as well as they raise ethical, logistical, and economic issues. In this study, we investigated the feasibility of specifically assessing the strength of human T-cell responses towards the kidneys of wild-type (WT) or transgenic pigs overexpressing human programmed death-1 ligand 1 (hPD-L1) during *ex vivo* kidney perfusion (EVKP). Human T cells were shown to adhere to the endothelium and transmigrate into WT and hPD-L1 kidneys. However, transcript levels of TNF-a and IFN-y as well as cytotoxic molecules such as granzyme B and perforin secreted by human T cells were significantly decreased in the tissue of hPD-L1 kidneys in comparison to WT kidneys. These results were confirmed via *in vitro* assays using renal endothelial cells (ECs) isolated from WT and hPD-L1 transgenic pigs. Both $CD4^+$ and $CD8^+$ T cells showed significantly lower proliferation rates after exposure to hPD-L1 porcine renal ECs in comparison to WT ECs. In addition, the secretion of pro-inflammatory cytokines was significantly reduced in cultures using hPD-L1 ECs in comparison to WT ECs. Remarkably, hPD-L1 EC survival was significantly increased in cytotoxic assays. This study demonstrates the feasibility of evaluating the human response of specific immune subsets such as human T

cells towards the whole xenograft during EVKP. This may represent a robust strategy to assess the potency of different genetic modifications to prevent xenogeneic immune responses and thereby predict the risk of immune rejection of new genetically engineered xenografts.

KEYWORDS

xenotransplantation, kidney transplantation, ex vivo organ perfusion, T-cell immune response, genetic engineering, programmed cell death-1 ligand 1 (PD-L1)

1 Introduction

Kidney transplantation represents the only curative treatment for patients with end-stage kidney failure (1). However, the availability of human kidneys suitable for transplantation is often associated with long periods on the transplant waiting lists. The discrepancy between the number of organs available and the increasing requirement for an organ presents a major obstacle and limits the number of successful kidney transplantations. On average, 18 patients from the waiting list die per day in Europe without receiving the chance of a life-prolonging organ (2).

Xenotransplantation provides a promising alternative to allogeneic transplantation by circumventing the bottleneck regarding available organs. However, despite similar organ size and physiology, genetic differences between species lead to immunological barriers and are a limiting factor for clinical success (1). The establishment of a variety of genetic modifications for xenotransplantation in combination with immunosuppressive and anti-inflammatory agents represents a promising approach to minimizing the risk of rejection (3). Transgenic pigs expressing human protective genes and knocked out for specific immune antigens significantly improved graft survival. In 2001, the first alpha-1,3-galactosyltransferase (GGTA1) deficient pigs were generated, providing a breakthrough success in xenotransplantation of porcine organs by reducing hyperacute rejection (HAR) (4–6). The generation of the first triple KO (GGTA1/Cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH)/β-1,4-acetyl-galactosaminyltransferase 2 (β4GalNT2)) pigs presented an additional milestone in overcoming HAR and acute humoral xenograft rejection (AHXR) (7). However, acute cellular rejection (ACR) remains the major obstacle to a successful xenotransplantation outcome and constitutes a considerable hurdle for long-term graft survival (8). In addition to the involvement of NK cells, macrophages, neutrophils, and B cells, T cells play a leading role in ACR (9, 10). T-cell receptor interaction with MHC, costimulation, and cytokine secretion regulate the activation of naïve T cells, which initiates a programmed differentiation pathway and determines the strength and functionality of the immune responses (11).

Organs of genetically engineered pigs might have the capacity to modulate human cellular immune responses and therefore represent a promising approach to support long-term xenograft survival. Attenuation of T-cell immune responses by preventing T-cell activation could demonstrate a benefit in reducing ACR (10). In cancer research, previous studies have shown that the binding of human programmed death-1 ligand 1 (hPD-L1) to the programmed death-1 (PD-1) receptor on T cells leads to the reduction of PD-1$^+$ cell proliferation, inhibition of cytokine secretion, and induction of apoptosis (12). In a xenogeneic setting, Buermann et al. indicated that hPD-L1 peripheral blood mononuclear cells (PBMCs) severe the potential to reduce CD4$^+$ T-cell proliferation and induce a low immunogenic, immune-protected status (13). However, the full effect of hPD-L1 overexpression on the porcine renal tissue in preventing human immune responses remains to be evaluated.

So far, potential human immune responses to xenografts carrying different combinations of genetic modifications can only be characterized *in vitro* using cultured human immune cells, in humanized mice models, or non-human primate (NHP) models after xenotransplantation (10, 14). However, the data generated using *in vitro* assays is often insufficient and reflects the immune response against a single target cell type and not against the complete tissue as in the case of transplantation. Studies based on humanized mouse models are strongly dependent on the degree of humanization and capacity to mount reliable immune responses. On the other hand, xenogenic immune responses can be successfully evaluated by transplanting NHPs in a preclinical state, but this strategy is associated with several ethical, logistic, and economic concerns (15, 16). Hence, precise assays enabling the assessment of specific human immune responses towards the xenograft in its complete multi-cell type and structure complexity are highly desirable. *Ex vivo* kidney perfusion (EVKP) has emerged as a promising technology for assessing the quality of kidneys during preservation and has also been shown to serve as a platform for organ conditioning, allowing targeted treatment and quality improvement (17). In this study, we evaluate the feasibilityof specifically assessing T-cell immune responses during EVKP, without the influence of other immune cells. This strategy may allow the characterization of the direct impact of specific genetic modifications in the T-cell immune response using a complete organ as a target and not only specific cell subtypes as in conventional *in vitro* assays. This strategy may enable an initial evaluation of the efficacy of specific genetic modifications and might

contribute to reduce and refine the number of animals used for the unavoidable preclinical tests on NHPs.

2 Materials and methods

2.1 Experimental groups and kidney retrieval

In this study, kidneys from 10 wildtype (WT) (non-perfused WT kidneys (n = 3), perfusions of WT kidneys without human T cells (n = 3), perfusion of WT kidneys with human T cells (n = 4)) and two genetically modified landrace pigs with GGTA1-KO and hPD-L1 overexpression were used. One kidney from each animal was used for perfusion. For organ retrieval, pigs were anesthetized with Propofol (i.v.) and euthanized with pentobarbital (i.v.). After circulatory death, an anterior midline incision was performed and rectus abdominis muscles were separated. The retroperitoneum and peri-renal space are exposed via blunt dissection. Following the dissection of the aorta and inferior vena cava, the kidneys were removed en bloc with these vessels. Kidneys were flushed with 200 mL cold (4°C) Custodiol (Dr. Franz Köhler Chemie GmbH, Bensheim, Germany) and stored on ice during transport to the laboratory.

2.2 Isolation of human T cells

PBMCs were isolated from human blood from different healthy donors immediately before the start of perfusion. Briefly, human blood was diluted 1:2 with Dulbecco's Phosphate Buffered Saline (Lonza, Basel, Switzerland) and centrifuged by density gradient centrifugation in Lymphosep (C. C. Pro, Oberdorba, Germany). Afterward, the $CD3^+$ cell population was isolated by negative magnetic bead isolation using the human Pan T Cell Isolation Kit (Miltenyi Biotec Inc., Auburn, California, USA) according to the manufacturer's instructions. In this study, $3.5x10^7$ T cells were used for perfusion.

2.3 Normothermic EVKP

Kidneys were connected to the Kidney Assist® perfusion device (XVIVO B.V., Groningen, Netherlands) via an artery cannula. After kidneys had been warmed up to 37°C for 30 minutes, organs were perfused for 4 hours with 1 L of Williams´s Media E (WME) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 7,15 g HEPES (Sigma Aldrich, Darmstadt, Germany), 50 g/L Bovine Serum Albumin (Sigma Aldrich) and 0,80 g Creatinine (Sigma Aldrich) as previously described (18, 19). T cells were injected into the perfusion system after the perfusate temperature reached 37°C. Perfusion flow, vascular resistance, oxygen saturation, and perfusate temperature were monitored every 30 minutes. After 270 minutes, EVKP was finished and kidneys were flushed with 1 L Custodiol. Non-perfused WT kidneys (n = 3), perfusions of WT kidneys without human T cells (n = 3), and perfusions only with T cells (n = 3) served as controls to WT (n = 4) and hPD-L1 kidneys (n = 2) perfused with T cells.

2.4 Histological evaluation

Immediately after perfusion, renal tissues were fixed in 4% paraformaldehyde and embedded in paraffin after 3 days. Tissue slices were stained with hematoxylin and eosin for analyses of renal structure. For immunohistochemistry, tissue slices were stained with anti-human CD3 (UCHT1; BioLegend, San Diego, USA) or CD274 Polyclonal Antibody (Bioss Antibodies, Woburn, Massachusetts, USA) by using the Zytochem Plus HRP Polymer System (Zytomed Systems, Berlin, Germany). Counterstaining was performed using Papanicolaou's solution and samples were fixed with DPX Mountant (Sigma-Aldrich, St. Louis, Missouri, USA). Afterward, visualization was performed using a Keyence microscope (Keyence, Itasca, Illinois, USA) and samples were quantified via QuPath v0.3.0 bioimage analysis software (open source; https://qupath.github.io/).

2.5 Perfusate analyses

2.5.1 Lactate dehydrogenase activity

Perfusate samples were collected at different time points (0, 30, 90, 150, 210, and 270 minutes) of perfusion. Lactate dehydrogenase (LDH) activity in perfusate samples was calculated using the colorimetric Cytotoxicity Detection Kit (LDH) (Roche, Basel, Switzerland) according to the manufacturer's instructions. The optical density (OD) of the colorimetric assay was used to determine the extent of LDH release.

2.5.2 Lactate levels

Perfusate samples were collected at different time points (0, 30, 90, 150, 210, and 270 minutes) after perfusion start to quantify lactate levels in the perfusate using the Lactate-Glo Assay System (Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol. For analysis, perfusate samples were diluted 1:50 with DPBS (Lonza), and relative luminescence units (RLU) were calculated using Lumat LB 9507 (Berthold Technologies, Zug, Switzerland) luminometer. L-lactate concentrations were measured by extrapolation using a standard curve.

2.6 Quantitative real-time polymerase chain reaction

Pooled tissues collected from 3 regions of the renal cortex and medulla (upper, lower, middle region) were fixed in RNAlater™ Stabilization Solution (Merck, Darmstadt, Germany) immediately after perfusion. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed to cDNA by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA). Transcript levels of human tumor necrosis factor-alpha (TNF-α) (Hs00174128_m1;

Thermo Fisher Scientific), interferon-gamma (IFN-γ) (Hs00194264_m1; Thermo Fisher Scientific), granzyme B (GZMB) (Hs00188051_m1; Thermo Fisher Scientific), and perforin (Hs00169473_m1; Thermo Fisher Scientific) were measured by utilizing TaqMan Gene Expression Master Mix (Thermo Fisher Scientific). All samples were analyzed in triplicates using the StepOnePlus Real-Time PCR system and results were evaluated by StepOnePlus Software v2.3 (Applied Biosystems). GAPDH (Hs02786624_g1, Thermo Fisher Scientific) was used as an endogenous control for the normalization of mRNA levels.

2.7 Isolation of renal endothelial cells

Biopsies were collected from three regions of the kidney (upper, lower, middle region) of unperfused WT or unperfused hPD-L1 renal cortex and medulla. Biopsies were pooled and digested with Collagenase Type I (Sigma-Aldrich) to obtain a single-cell suspension. Cells were cultured in endothelial cell growth medium (EGM-2) (Lonza) on gelatin-coated plates. EC phenotyping was performed by analyzing CD31 and CD144 expression using APC/Cyanine7 anti-human CD31 (WM59; BioLegend) and Alexa Fluor® 647 mouse anti-human CD144 (55-7H1; BD Biosciences, Franklin Lakes, New Jersey, USA) antibodies. PD-L1 expression was evaluated using APC anti-human CD274 antibody (29E.2A3; BioLegend). Data were evaluated by BD FACSCanto™ II Clinical Flow Cytometer System (BD Biosciences) and results were analyzed using FlowJo software v10.6.2 (BD Biosciences).

2.8 Human T-cell proliferation assay

24 hours before the start of cell co-culturing (day 0), $2x10^4$ target cells (WT and hPD-L1 ECs) were seeded in triplicates onto a 96-well plate. On day 1, $2x10^5$ T cells from four healthy donors were labeled with the cell proliferation dye efluor 670 (Thermo Fischer Scientific). Afterward, T cells were added in a 10:1 (E: T) ratio to the ECs and cultured in RPMI 1640 Medium (Lonza) supplemented with 5% AB serum (c.c.pro GmbH) and interleukin (IL)-2 (100 and U/mL; Prepotech, New Jersey, USA). After 7 days of co-culturing, the experiment was finished and T cells were stained with FITC anti-human CD3 antibody (UCHT1; BioLegend), APC/Cyanine7 anti-human CD4 antibody (SK3; BioLegend), and PE anti-human CD8 antibody (SK1; BioLegend). T-cell proliferation rates were evaluated by comparing proliferation values of day 0 with day 7 using BD FACSCanto™ II Clinical Flow Cytometer System (BD Biosciences) and results were analyzed using FlowJo software v10.6.2 (BD Biosciences).

2.9 Real-time cytotoxicity assay

24 hours before the experiment started, $2x10^4$ target cells (WT and hPD-L1 ECs) were seeded in duplicates onto microtiter plates (E-Plates©; Agilent Technologies, California, USA). After achieving a confluent EC monolayer, $2x10^5$ T cells were isolated from three different donors and added in a ratio of 10:1 (E:T) to the target cells. Cells were cultured in RPMI 1640 medium (Lonza) supplemented with 5% human serum AB (c.c.pro GmbH) and IL-2 (100 U/mL) (Prepotech) for 140 hours. Cell proliferation as a function of real-time changes in electrical impedance, also referred to as cell index, was continuously monitored using the xCELLigence Real-Time Cell Analyzer (Agilent Technologies).

2.10 Cytokine multiplex analyses

Pro-inflammatory cytokine profile indicating a xenogenetic T-cell response was determined in the real-time cytotoxicity assay´s supernatant using the MILLIPLEX Human Cytokine/Chemokine/Growth Factor Panel A Magnetic Bead Panel (Merck KGaA, Darmstadt, Germany). Briefly, secretion levels of human IFN-γ, IL-8, interferon gamma-induced protein 10 (IP-10), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-5, IL-10, IL-1b, and IL12p70 were quantified in 25 µL centrifuged perfusate samples collected at the end of the assay (time point 140 hours) and measured using Luminex® 100/200 analyzer (Luminex Corp., Austin, Texas, USA). Standard and sample preparations were performed according to the manufacturer's instructions. Cytokine levels were calculated using the Xponent software version 3.1 (Luminex Corp.).

2.11 Statistical analyses

All data are presented as mean ± standard deviation. For comparison between two groups, the student's t-test was used. One-way ANOVA with multiple comparisons was applied to compare data with one variable between more than two groups. Two-way ANOVA was used for comparisons of data with two categorical variables between more than two groups. $p < 0.05$ were considered significant and defined as $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. All statistical analyses were performed using GraphPad Prism version 8 software (GraphPad Software Inc, San Diego, California, USA).

3 Results

3.1 hPD-L1 expression on renal tissue of WT and transgenic pigs

ECs play crucial roles during graft rejection by several mechanisms including antigen presentation to circulating T cells, or by triggering inflammatory processes and thrombosis (20). Therefore, we confirmed the overexpression of hPD-L1 on renal ECs isolated from the transgenic pigs. Expression of typical markers such as CD31 and CD144 on isolated WT (CD31: 93.30 ± 4.67%; $CD31^+$ $CD144^+$: 75.37 ± 14.06%) and hPD-L1 ECs (CD31: 99.86 ± 0.19%; $CD31^+$ $CD144^+$: 46.47 ± 10.71%) showed no significant

differences between the groups (Figures 1A, B). Remarkably, flow cytometry analyses revealed significantly ($p < 0.0001$) increased PD-L1 expression on hPD-L1 (MFI: 4832.50 ± 11.50) in comparison to WT (MFI: 342.00 ± 80.70) ECs (Figures 1C, D). Accordingly, immunohistochemically quantification showed significantly ($p < 0.01$) increased PD-L1 expression in the renal tissue of hPD-L1 (94.94 ± 2.15%) in comparison to WT (45.13 ± 9.17%) pig-derived tissues (Figures 1E, F).

3.2 Effect of T cells on EVKP parameters

EVKP represents a promising opportunity to provide optimal organ preservation and quality assessment between organ retrieval and transplantation (17). In this study, we specifically evaluated *ex vivo* human T-cell immune responses targeting the renal endothelium during normothermic EVKP as an alternative method to assessment after transplantation. After a 30-minute warm-up period of the perfusion solution to 37°C, the perfusion temperature was kept constant at normothermic temperatures of 36-37°C for the entire perfusion period. WT kidneys perfused without T cells, WT kidneys perfused with T cells, and hPD-L1 kidneys perfused with T cells reached average flow rates of 158.93 ± 27.75 mL/min, 153.34 ± 15.35 mL/min, and 145.45 ± 19.93 mL/min, respectively, with corresponding vascular resistance (VR) values of 0.22 ± 0.15 mmHg/mL/min, 0.25 ± 0.05 mmHg/mL/min, and 0.21 ± 0.07 mmHg/mL/min. During the entire perfusion, oxygen saturation in the perfusate was maintained at constant values of 81.41 ± 4.06%, 81.94 ± 2.10%, and 80.01 ± 1.92%, respectively. Despite initial variations in perfusion parameters during the warm-up phase, no significant differences were observed in flow rate, VR, perfusion temperature, and oxygen saturation during normothermic EVKP (Figures 2A–F).

3.3 Perfusion with human T cells does not induce tissue damage

Histological analyses were performed to evaluate tissue integrity after EVKP. The histopathological findings suggested no significant difference between kidney perfusions with and without T cells, or between T-cell perfusions of WT and hPD-L1 kidneys. However, all perfused kidneys exhibited mild dilatation of Bowman's capsule and potentially reversible moderate acute intratubular injury with overall intact renal morphology (Figures 3A–D).

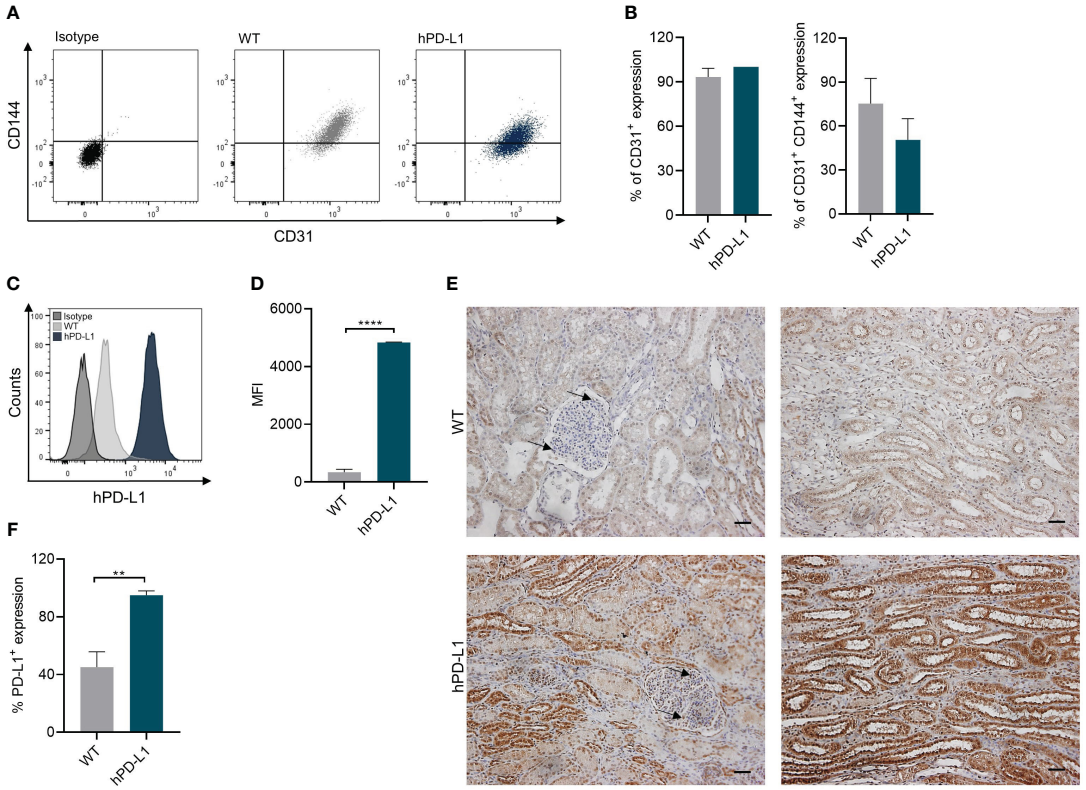


FIGURE 1
Endothelial cell (EC) isolation from WT and hPD-L1 kidneys. **(A)** Representative dot plots of $CD31^+$ and $CD144^+$ expression on renal ECs. **(B)** Mean and standard deviation of $CD31^+$ and $CD31^+$ $CD144^+$ expression on ECs isolated from WT ($n = 3$) and hPD-L1 ($n = 2$) kidneys. **(C)** Representative histogram shows PD-L1 expression on WT and hPD-L1 ECs. **(D)** MFI and standard deviation of PD-L1 expression on ECs (WT: $n = 3$; hPD-L1: $n = 2$). **(E)** Immunohistochemistry staining demonstrated representative PD-L1 expression on WT and hPD-L1 kidney tissues (Scale bar: 50µm). Arrows indicate endothelial cells. **(F)** Mean percentage and standard deviation of PD-L1 expression on immunohistochemistry stained tissues (WT: $n = 4$; hPD-L1: $n = 2$). Statistical significance was evaluated using an unpaired t-test (**p < 0.01; ****p < 0.0001).

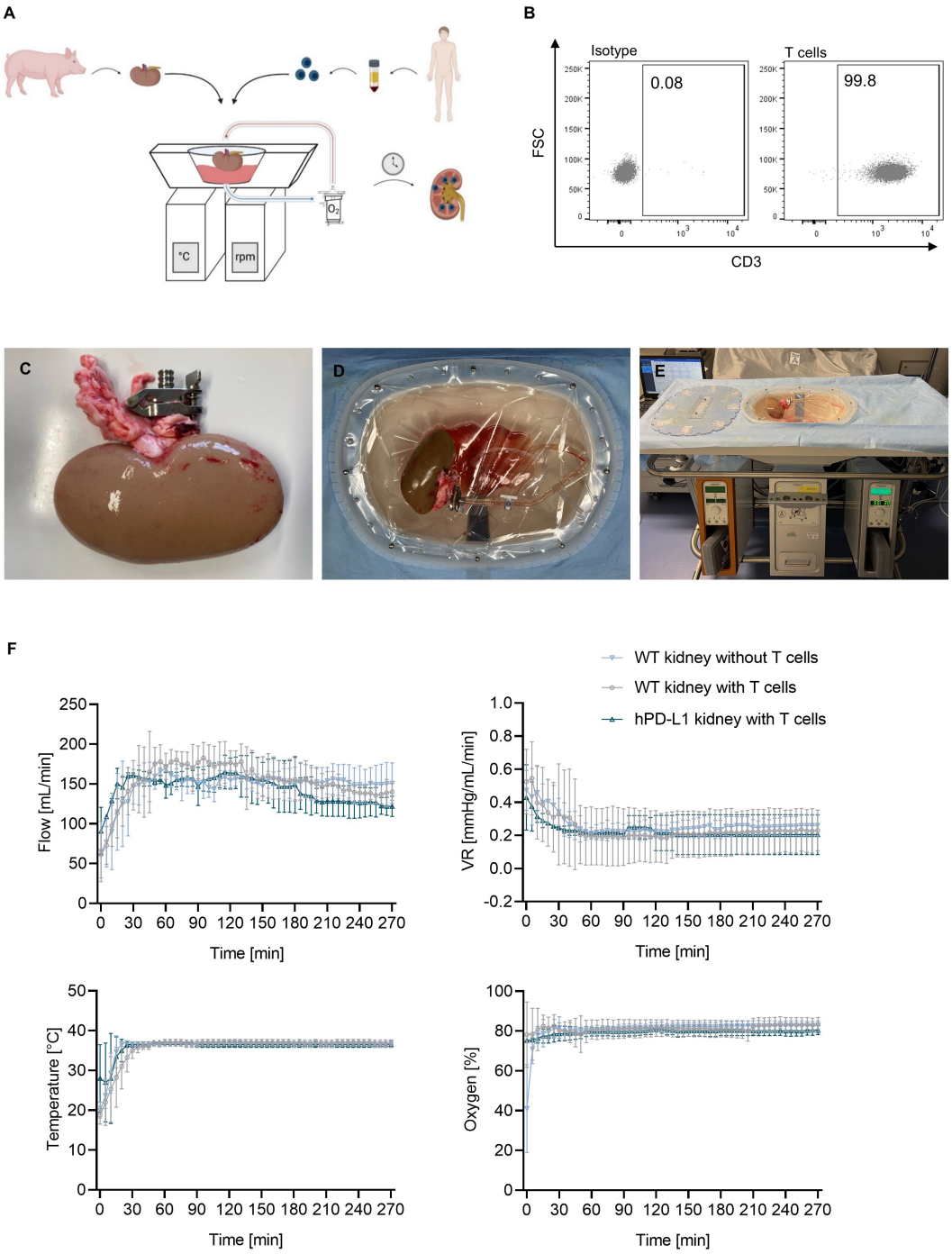


FIGURE 2
Normothermic *ex vivo* kidney perfusion (EVKP) with human T cells. **(A)** Schematic representation of the EVKP system. The figure illustrates the kidney retrieval from a donor pig, the isolation of the T cells from human blood, and the main components of the perfusion system (perfusion reservoir, thermo unit, pump unit, oxygenator). **(B)** Representative dot plots show $CD3^+$ expression on human T cells after isolation. **(C–E)** Representative pictures of the kidney during perfusion. **(C)** Picture displays the clamp placed in the renal artery, **(D)** the arterial connection of the kidney to the perfusion system, and **(E)** the kidney perfusion system. **(F)** Graphs display the monitored flow rate, vascular resistance (VR), temperature, and oxygen partial pressure of wildtype (WT) kidney perfusions without T cells (n = 3), WT kidney perfusions with T cells (n = 4), and programmed death ligand-1 (hPD-L1) kidney perfusions with T cells (n = 2). Graphs show means and standard deviations.

LDH activity levels are considered a marker to assess tissue integrity (21, 22). During EVKP, no significant differences in LDH levels were observed in perfusion solution of kidney perfusions without T cells (T_{270}: 0.87 ± 0.10), WT kidney perfusions with T cells (T_{270}: 1.04 ± 0.20), and hPD-L1 kidney perfusions with T cells (T_{270}: 0.72 ± 0.14) compared to the LDH activity level absence detected in perfusions only with T cells (Figure 3E).

Lactate levels are commonly used as a marker to evaluate tissue integrity and indicate signs of acute injury (23). An

10.3389/fimmu.2024.1279050

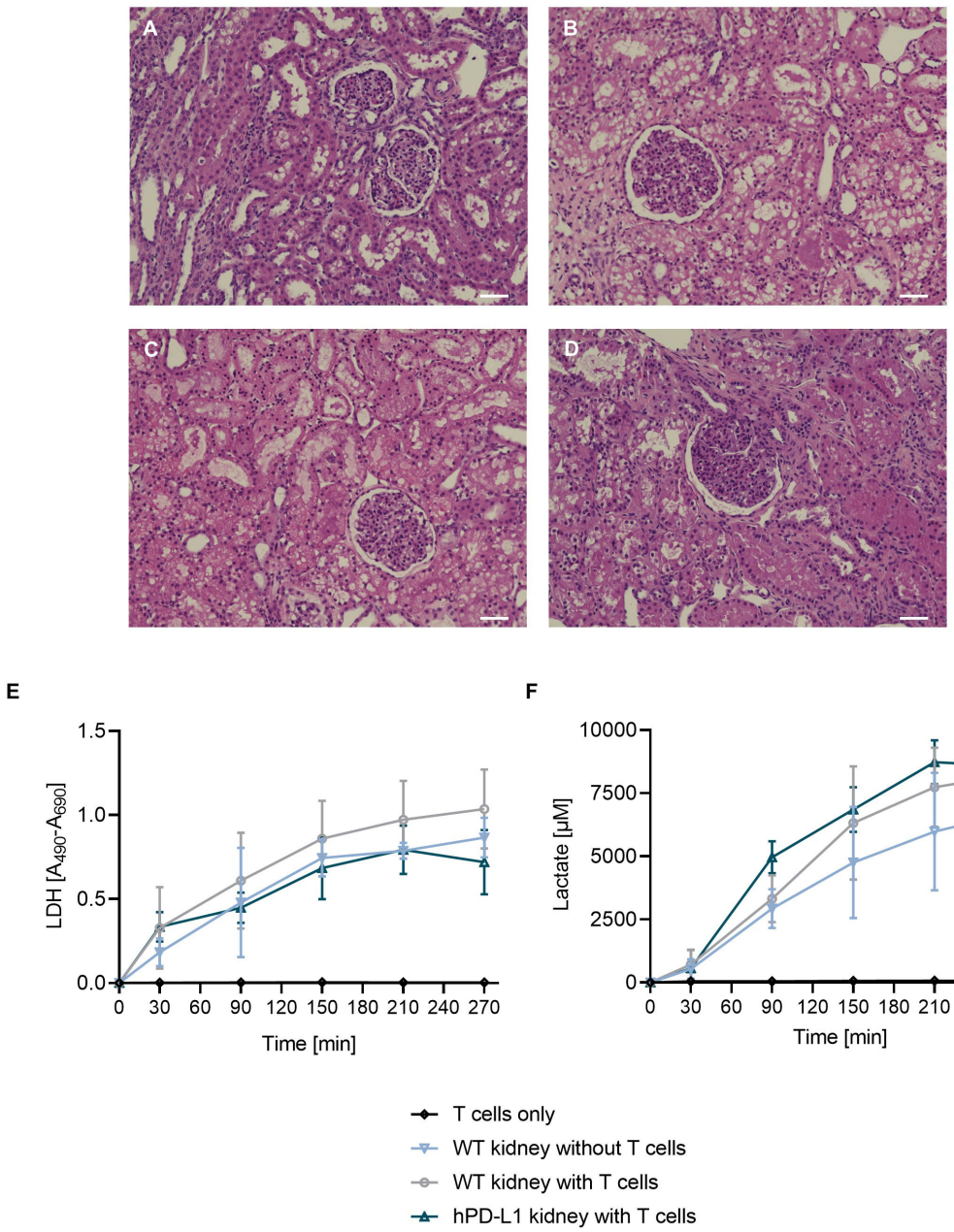


FIGURE 3
Quality assessment of the kidney after EVKP. **(A–D)** Histological analysis of kidneys after EVKP. Representative pictures of hematoxylin and eosin-stained renal cortex: **(A)** unperfused WT kidney, **(B)** WT kidney perfused without T cells, **(C)** WT kidney perfused with xenoreactive human T cells, and **(D)** hPD-L1 kidney perfused with xenoreactive T cells. Scale bar 50 µm. **(E)** Lactate dehydrogenase (LDH) activity and **(F)** lactate levels were quantified in perfusates at different time points (0, 30, 90, 150, 210, and 270 minutes). Graphs represent means and standard deviations of perfusions only with T cells (n = 3), WT kidney perfusions without T cells (n = 4), WT kidney perfusions with T cells (n = 3), and hPD-L1 kidney perfusions with T cells (n = 2).

increase in the lactate concentration in the perfusion solution was detected during the perfusion of WT kidneys without T cells as well as WT and hPD-L1 kidney perfusions with T cells. As expected, no lactate increase could be detected in control runs only with T cells over time. In contrast, lactate concentrations of kidney perfusions without T cells (T_{270}: 6756.84 ± 1461.59 µM), WT kidney perfusions with T cells (T_{270}: 8260.99 ± 1025.62 µM), and hPD-L1 kidney perfusions with T cells (T_{270}: 8272.83 ± 50.13 µM) increased throughout the perfusion time (Figure 3F). The results suggest that xenogeneic T cells do not significantly affect kidney integrity or tissue injury during EVKP.

3.4 hPD-L1 ECs induce weaker xenogeneic T-cell immune responses

Immunohistochemical analyses of perfused tissue allowed the detection of T-cell transmigration into the tissue. Whereas no T-cell

10.3389/fimmu.2024.1279050

infiltration occurred in the tissue perfused without T cells, $CD3^+$ cells were detected in the tissue perfused with T cells from both WT and hPD-L1 kidneys after the end of the perfusion. The transmigrated T cells were predominantly localized in the renal tubule after four hours of perfusion, however, individual T cells had already infiltrated into the tissue (Figure 4A). This data shows that during EVKP, T cells are capable to adhere and transmigrate into the renal tissue where they might respond to it.

The transcript levels of human cytokines including TNF-α and IFN-γ, as well as GZMB, and perforin were measured to investigate xenogeneic T-cell responses. Human T cells were demonstrated to respond specifically to porcine kidney tissue, as evidenced by increased T-cell transcript levels of cytokines and cytotoxic molecules in perfused WT and hPD-L1 porcine kidney tissues compared to porcine tissues perfused without T cells, where they were not detectable. TNF-α, IFN-γ, GZMB, and perforin transcript levels of hPD-L1 kidneys were reduced by 70.08 ± 4.94% ($p < 0.01$), 40.35 ± 8.00% ($p < 0.01$), 48.92 ± 6.35% ($p < 0.001$), and 65.03 ± 4.15% (non-significantly), respectively, in comparison to WT kidneys (99.99 ± 31.59%, 99.96 ± 16.78%, 99.96 ± 12.13%, 99.95 ± 74.83%) (Figure 4B). These data suggest that hPD-L1 kidneys may induce weaker xenogeneic T-cell immune responses.

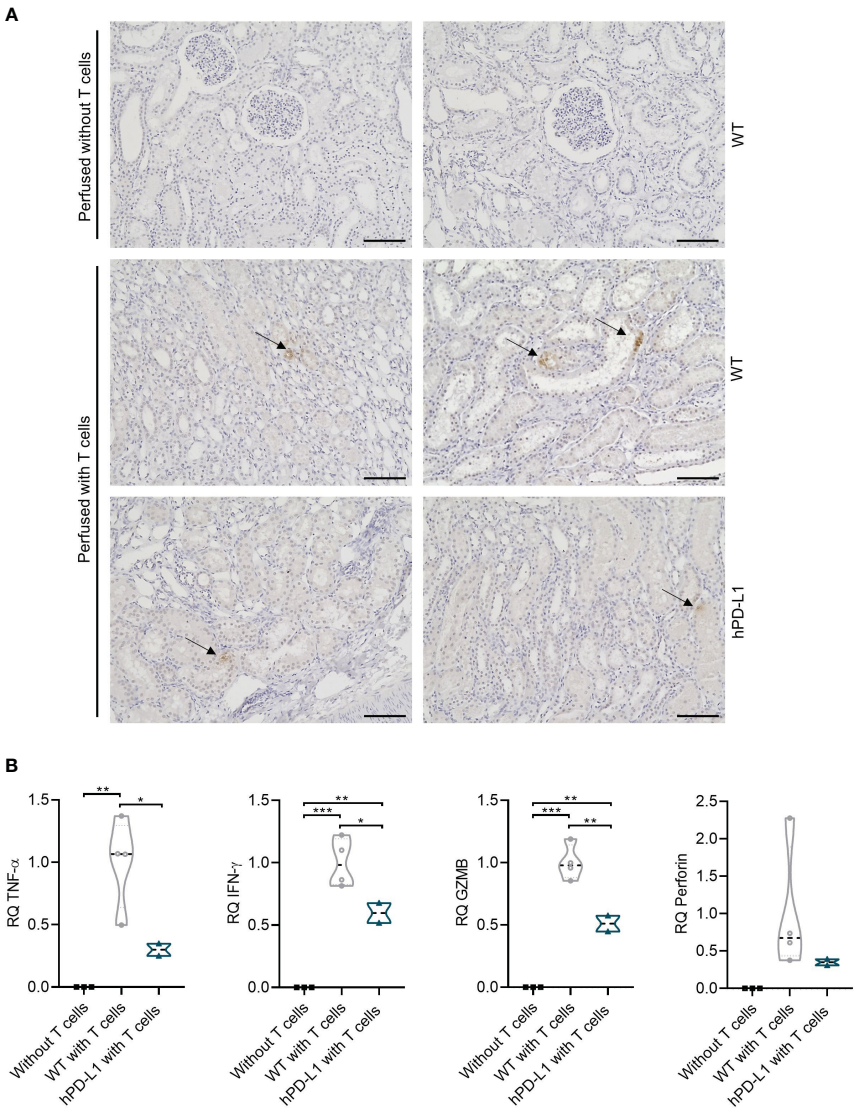


FIGURE 4
Evaluation of T-cell infiltration in renal tissue and assessment of xenogeneic T-cell immune response after EVKP. **(A)** $CD3^+$ immunohistochemistry staining of the perfused kidney without T cells and with human T cells (WT and hPD-L1) after the end of perfusion. Arrows point to the infiltrated T cells (Scale bar: 100µm). **(B)** Relative quantification (RQ) of tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), granzyme B (GZMB), and perforin transcript levels detected in kidney tissue perfused without T cells ($n = 3$), WT kidney tissue perfused with T cells ($n = 4$), and hPD-L1 kidney tissue perfused with T cells ($n = 2$). Statistical significance was evaluated using one-way ANOVA (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

3.5 hPD-L1 overexpression on porcine ECs shows a protective effect against xenogeneic T-cell responses

Antigen-specific immune responses are usually associated with an increase in T-cell proliferation rates (24). We have assessed the capacity of human helper or cytotoxic T-cell subpopulations to proliferate after exposition to EC isolated from WT or hPD-L1 pigs. In this xenogeneic setup, significantly reduced $CD4^+$ helper and $CD8^+$ cytotoxic T-cell proliferation was observed when hPD-L1 ECs were analyzed ($CD4^+$: 16.38 ± 0.96%, $p < 0.05$; $CD8^+$: 51.10 ± 4.70%, $p < 0.01$) in comparison to the proliferation rates detected with WT ECs ($CD4^+$: 23.80 ± 5.09%; $CD8^+$: 68.08 ± 4.21). A similar effect was observed in cultures using both $CD4^+$ and $CD8^+$ T cells (i.e. $CD3^+$ T-cell populations). While only 26.68 ± 1.26 ($p < 0.0001$) of $CD3^+$ cells proliferated in the presence of hPD-L1 ECs, 40.78 ± 2.12% $CD3^+$ cells proliferated after exposure to WT ECs (Figures 5A, B).

Cytokines are important mediators of immune responses after transplantation (25). Levels of cytokines detected in the cell culture supernatants of T cells incubated with hPD-L1 ECs were lower than those measured in WT ECs: IL-1β: 6.45 ± 8.47 ng/μL vs. 0.98 ± 1.38 ng/μL, IL-5: 37.97 ± 17.33 ng/μL vs. 6.91 ± 4.29 ng/μL, IL-8: 2368.43 ± 2456.10 ng/μL vs. 497.71 ± 501.51 ng/μL; IL-10: 28.92 ± 23.55 ng/μL vs. 9.79 ± 7.31 ng/μL; IL-12p70: 2.32 ± 1.60 ng/μL vs. 2.19 ± 2.48 ng/μL; GM-CSF: 1839.99 ± 798.28 ng/μL vs. 360.34 ± 267.17 ng/μL; IFN-γ: 1838.99 ± 798.28 ng/μL vs. 360.34 ± 268.17 ng/μL, and IP-10: 1720.55 ± 955.90 ng/μL vs. 1004.77 ± 1002.40 ng/μL (Figure 5C).

In addition, assessment of T-cell-mediated cytotoxicity is critical for evaluating the capacity of the xenogeneic immune response to injure the graft, as even a small number of T cells may lead to organ rejection (26, 27). Compared to WT ECs, hPD-L1 ECs co-cultured with human T cells exhibited higher survival rates (CI, Cell index) over time (140 hours). After 30 hours, the survival rate of hPD-L1 ECs (CI: 0.91 ± 0.04, $p < 0.05$) was significantly increased in comparison to WT ECs (CI: 0.46 ± 0.11). This effect was even more pronounced after 60 hours (hPD-L1 $CI_{60\text{-}140}$: 0.98 ± 0.23 vs. $WTCI_{60\text{-}140}$: 0.09 ± 0.07, $p < 0.0001$) (Figure 5D). Altogether, these data indicate that hPD-L1 overexpression on renal endothelial cells induces significantly weaker immune responses and results in protection against T-cell xeno-cytotoxicity.

4 Discussion

Xenotransplantation of porcine organs represents a promising approach to circumvent the shortage of human organs available for transplantation. Breakthrough advances in the field of xenotransplantation have been made by the revolutionized CRISPR-Cas9 technology, which allows the development of multigene-modified pigs such as triple KO (GGTA1/CMAH/β4GalNT2) pigs to overcome HAR and AHXR (28). In 2021, the first kidney xenotransplantations from pig-to-human were performed. Two genetically modified kidneys were transplanted into brain-dead patients observing graft survival of 54 hours (29). In January 2022, the first heart xenotransplantation from pig-to-human was performed. The patient survived two months with the xenogeneic transplant (30).

Currently, the pre-clinical evaluation of the efficiency of novel genetic modifications introduced to pigs in preventing human immune responses relies on the use of NHPs. However, on the one hand, the application of NHPs is associated with relevant ethical and moral evaluation in terms of social, health, religious, legal, and regulatory considerations (15), and on the other hand, NHPs do not represent identically pig-to-human coagulation and immune responses due to the species intrinsic genetic differences. First, macaques carry a "hypercoagulable" phenotype, which can lead to increased coagulopathy compared to humans. Also in contrast to humans, NHP and pigs express the N-glycolylneuraminic acid (Neu5Gc) and therefore NHP does not form specific anti-pig Neu5Gc antibodies as it occurs after xenotransplantation in humans (31). The application of *in vitro* immunological assays to establish and test novel genetic modifications is highly desirable. However, such assays often focus on the use of a single cell type and do not represent the level of organ complexity in cell composition and 3D structure, making them prone to deliver insufficient data.

EVKP emerged as a novel strategy for organ preservation with the potential to reduce storage damage, improve graft assessment, and potentially contribute to graft survival after transplantation (32). EVKP allows the maintenance of the organ under physiological parameters supported by continuous oxygen delivery, and pulsatile flow through the renal vasculature at normothermic conditions (33, 34). These physiological environments were shown to be appropriate for evaluating the human xenogeneic T-cell immune response *ex vivo*. This assay may be used as a first assessment of the T-cell response towards genetically engineered pig kidneys not only to elucidate cellular and molecular mechanisms but also to allow the reduction of animals and refinement of NHP pre-clinical studies. It should be mentioned, that this strategy alone is not sufficient to replace the preclinical studies, as they provide further indispensable results in terms of longer evaluation time, graft function, and safety.

Recently, *ex vivo* organ perfusion (EVOP) has gained plenty of attention as a model to evaluate human xenogeneic immune response. Previous studies using *ex vivo* heart perfusion with human whole blood showed an increase of cytokines such as IL-2, IL-4, and IFN-γ as well as cytotoxic molecule secretion such as GZMB and perforin by T-cell subsets (35). Moreover, pig kidneys have been perfused with human whole blood (36) or human peripheral blood lymphocytes (37). In this study, we used EVKP as a model perfusing porcine kidneys with freshly isolated human T cells to assess the pig-to-human T cell-mediated xenogeneic immune responses. This allows us to evaluate precisely the impact of specific genetic modifications in the pig kidney on human xenogeneic T-cell responses without the interference of other immune cell subpopulations that might have been activated due to the perfusion conditions.

10.3389/fimmu.2024.1279050

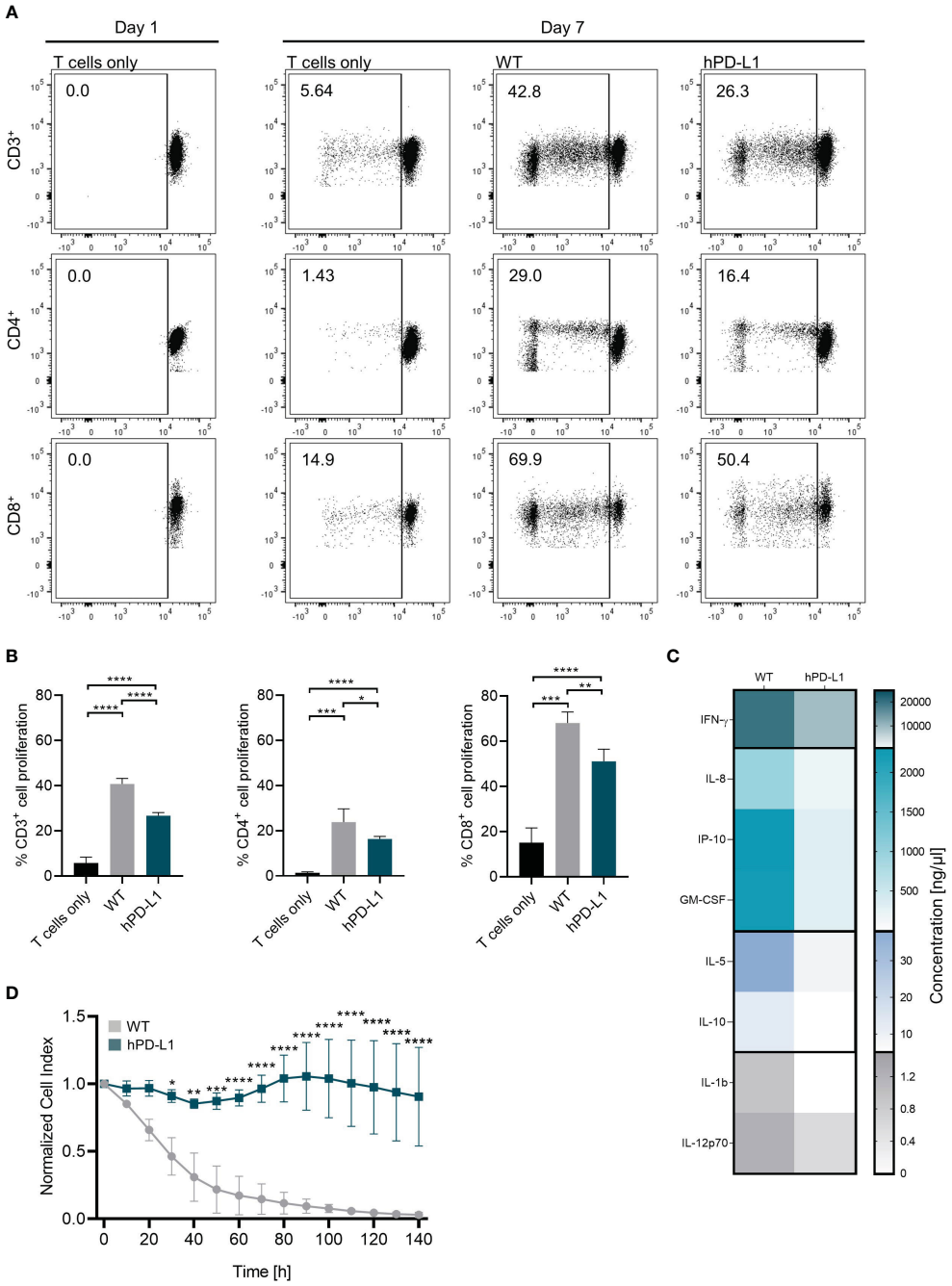


FIGURE 5
hPD-L1 overexpression on porcine ECs shows a protective effect against xenogeneic T-cell responses. **(A)** Representation of $CD3^+$, $CD4^+$, and $CD8^+$ T-cell proliferation on day 1 and day 7. Proliferation rates of T cells co-cultured without target cells (T cells only) and xenoreactive human T cells isolated from four donors co-cultured with WT and hPD-L1 ECs were evaluated. **(B)** Mean and standard deviation of $CD3^+$, $CD4^+$, and $CD8^+$ T-cell proliferation of T cells alone or exposed to WT and hPD-L1 ECs. Statistical significance was evaluated using one-way ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$). **(C)** Heat map represents cytokine release profile after exposure of human T cells to WT and hPD-L1 ECs for 140 hours. Color saturation represents the average values of the concentrations ($n = 3$) of IFN-γ, interleukin 8 (IL-8), interferon gamma-induced protein 10 (IP-10), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-5, IL-10, IL-1b, and IL-12p70. **(D)** Normalized cell index of WT and hPD-L1 ECs incubated for 6 days with human T cells ($n = 3$). Statistical significance was evaluated using two-way ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

The graft endothelium is the first immune checkpoint between the recipient's immune system and the renal graft (38). However, recent studies also indicate that renal proximal tubular epithelial cells may also be directly recognized by T cells, which may immediately contribute to rejection (39, 40). Our immunohistochemistry analyses after EVKP indicate the presence of human T-cell focal adhesions in different kidney regions where the recognition of xenoantigens may occur. Our results are consistent with previous studies in rats examining the levels of T-cell infiltration after transplantation of PD-L1-expressing porcine B

cells under the renal capsule. Compared to the mock-transfected control cells, which showed scattered to moderate T-cell infiltration after 7 days, the number of infiltrated T cells remained low in the rats transplanted with hPD-L1 cells (41).

To assess the strength of the T-cell immune responses we have evaluated the up-regulation of immunomodulators and cytotoxic molecules. Cytokines such as TNF-α or IFN-γ play a crucial role in the processes such as immune modulation and inflammatory response. TNF-α is both a pro-inflammatory and an anti-inflammatory cytokine secreted by effector $CD4^+$ and $CD8^+$ T cells (42, 43). Low TNF-α concentrations contribute to cell survival, differentiation, and proliferation, however, excessive activation of TNF-α signaling is often associated with chronic inflammation (44). IFN-γ is secreted by T-helper cells and contributes to the activation of macrophages (45). Perforin and granzymes are important effector molecules of cytotoxic T cell-mediated cell death. The cytotoxic granules of T cells contain the pore-forming protein perforin and serine proteases (granzymes) (46), which can be considered markers for rejection (47). Accordingly, this study demonstrated the feasibility of assessing the activation of T cells by the pig kidney based on the upregulation of TNF-α, IFN-γ, GZMB, and perforin transcript levels during EVKP. Remarkably, we could demonstrate that transcript levels of those molecules were downregulated during hPD-L1 kidney perfusion compared to the WT kidneys, suggesting a potential protective effect of hPD-L1 against acute cellular rejection mediated by xenoreactive T cells.

The value of evaluating human T-cell responses during EVKP relies on the feasibility of assessing T-cell recognition, activation, and functionality in a nearly physiological setup. However, several parameters associated with the perfusion such as pressure or flow rates may influence the T-cell response towards the organ. Furthermore, a limitation in the assessment of the feasibility of evaluating such responses was the number of hPD-L1 transgenic pigs available for this study. Therefore, we performed additional *in vitro* studies to confirm the results obtained during EVKP with human T cells.

Previous studies have indicated that PD-L1 overexpression inhibits the proliferation of human xenogeneic $CD4^+$ T cells and induces T-cell apoptosis (8, 13, 48, 49). Accordingly, our results showed reduced $CD4^+$ T-cell proliferation rates after exposure to porcine renal ECs. In addition, reduced $CD8^+$ T-cell proliferation was also observed.

Previously, we and others have shown that the cytokine secretion profile of T cells is associated with the strength of their response to target cells. In addition, secretion of pro-inflammatory cytokines such as IFN-γ or IL-1β was shown to correlate with kidney rejection. Also, in the pig-to-primate xenotransplantation setting, specific cytokines such as IFN-γ were demonstrated to be relevant systemic inflammatory factors that might contribute to the loss of xenograft function (50–52). On the other hand, decreased levels of cytokines such as IL-6, IL-10, IL-12, TNF-α, and IFN-γ have been associated with increased immunologic tolerance, reduced risk of acute rejection, and thus prolonged graft survival (53, 54). Our results indicate that the overexpression of hPD-L1 in porcine renal ECs significantly decreases the T-cell cytokine secretion. This suggests that the overexpression of hPD-L1 might contribute to xenograft survival.

PD-L1 expression is known to compromise T cell-mediated cytotoxicity against tumors (55). In the field of xenotransplantation, *in vitro* studies using a porcine B cell line overexpressing PD-L1 were shown to induce lower T-cell activation and cytotoxicity (48). Interestingly, in the pig-to-rat cell transplantation model, overexpression of PD-L1 was also demonstrated to induce weaker antibody-mediated immune responses (41). Our results using ECs isolated from the kidneys of hPD-L1 transgenic pigs confirmed that the overexpression of hPD-L1 contributes to the attenuation of T-cell cytotoxicity.

Unfortunately, α-Gal knockout pig kidneys were not available for this study and we only had access to two PD-L1 kidneys. Nevertheless, this study showed the feasibility of investigating T-cell immune responses during EVKP and that by using two types of kidneys (WT vs. PD-L1) alteration in the strength of the immune responses could be detectable. Furthermore, the responses observed during EVKP were in accordance with previously published data and with the results obtained during the *in vitro* assays performed in this study.

In summary, we represented the feasibility of evaluating the strength of human xenogeneic T-cell immune responses towards the pig kidney in its original complexity at cellular and structural levels during EVOP. Remarkably, we demonstrated that the immunogenicity of hPD-L1 kidneys for xenoreactive T cells was reduced compared with WT kidneys in both EVKP and *in vitro* assays. Hence, EVOP may be used in the future as a robust platform, ethically justifiable, and cost-effective approach to investigate additional genetic modifications that might contribute to the success of xenotransplantation.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Ethic Committee of the Hannover Medical School. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by Niedersächsische Landesamt für Verbraucherschutz und Lebensmittelsicherheit. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

KS: Conceptualization, Formal analysis, Data curation, Investigation, Methodology, Visualization, Writing – original draft. TR: Writing – review & editing. SB: Investigation, Writing – review & editing. RS: Supervision, Writing – review & editing, Investigation. RB: Resources, Writing – review & editing. BP:

Resources, Conceptualization, Writing – review & editing. CF: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was supported by Transregional Collaborative Research Centre 127 (TRR127-Biology of xenogeneic cells, tissue and organ transplantation-from bench to bedside).

Acknowledgments

We are thankful to Susanne Aufderbeck, Yvonne Speidel, and Beata Meyer for the excellent technical assistance.

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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

References

1. Lu T, Yang B, Wang R, Qin C. Xenotransplantation: current status in preclinical research. *Front Immunol* (2019) 10:3060. doi: 10.3389/fimmu.2019.03060

2. Hryhorowicz M, Zeyland J, Słomski R, Lipiński D. Genetically modified pigs as organ donors for xenotransplantation. *Mol Biotechnol* (2017) 59(9-10):435–44. doi: 10.1007/s12033-017-0024-9

3. Cooper DKC, Ekser B, Tector AJ. Immunobiological barriers to xenotransplantation. *Int J Surg* (2015) 23(Pt B):211–6. doi: 10.1016/j.ijsu.2015.06.068

4. Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, et al. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science* (2002) 295(5557):1089–92. doi: 10.1126/science.1068228

5. Dai Y, Vaught TD, Boone J, Chen SH, Phelps CJ, Ball S, et al. Targeted disruption of the alpha1,3-galactosyltransferase gene in cloned pigs. *Nat Biotechnol* (2002) 20 (3):251–5. doi: 10.1038/nbt0302-251

6. Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, et al. Production of alpha 1,3-galactosyltransferase-deficient pigs. *Science* (2003) 299(5605):411–4. doi: 10.1126/science.1078942

7. Estrada JL, Martens G, Li P, Adams A, Newell KA, Ford ML, et al. Evaluation of human and non-human primate antibody binding to pig cells lacking GGTA1/CMAH/ β4GalNT2 genes. *Xenotransplantation* (2015) 22(3):194–202. doi: 10.1111/xen.12161

8. Plege A, Borns K, Baars W, Schwinzer R. Suppression of human T-cell activation and expansion of regulatory T cells by pig cells overexpressing PD-ligands. *Transplantation* (2009) 87(7):975–82. doi: 10.1097/TP.0b013e31819c85e8

9. Kemter E, Schnieke A, Fischer K, Cowan PJ, Wolf E. Xeno-organ donor pigs with multiple genetic modifications - the more the better? *Curr Opin Genet Dev* (2020) 64:60–5. doi: 10.1016/j.gde.2020.05.034

10. Carrier AN, Verma A, Mohiuddin M, Pascual M, Muller YD, Longchamp A, et al. Xenotransplantation: A new era. *Front Immunol* (2022) 13:900594. doi: 10.3389/ fimmu.2022.900594

11. Priyadharshini B, Greiner DL, Brehm MA. T-cell activation and transplantation tolerance. *Transplant Rev (Orlando)* (2012) 26(3):212–22. doi: 10.1016/j.trre.2011.09.002

12. Han Y, Liu D, Li L. PD-1/PD-L1 pathway: current researches in cancer. *Am J Cancer Res* (2020) 10(3):727–42.

13. Buermann A, Petkov S, Petersen B, Hein R, Lucas-Hahn A, Baars W, et al. Pigs expressing the human inhibitory ligand PD-L1 (CD 274) provide a new source of xenogeneic cells and tissues with low immunogenic properties. *Xenotransplantation* (2018) 25(5):e12387. doi: 10.1111/xen.12387

14. Yamamoto T, Hara H, Iwase H, Jagdale A, Bikhet MH, Morsi MA, et al. The final obstacle to successful pre-clinical xenotransplantation? *Xenotransplantation* (2020) 27 (5):e12596. doi: 10.1111/xen.12596

15. Cengiz N, Wareham CS. Ethical considerations in xenotransplantation: a review. *Curr Opin Organ Transplant* (2020) 25(5):483–8. doi: 10.1097/MOT.0000000000000796

16. Langley G, Evans T, Holgate ST, Jones A. Replacing animal experiments: choices, chances and challenges. *Bioessays* (2007) 29(9):918–26. doi: 10.1002/bies.20628

17. Zulpaite R, Miknevicius P, Leber B, Strupas K, Stiegler P, Schemmer P. Ex-vivo kidney machine perfusion: therapeutic potential. *Front Med (Lausanne)* (2021) 8:808719. doi: 10.3389/fmed.2021.808719

18. Mahboub P, Ottens P, Seelen M, t Hart N, Van Goor H, Ploeg R, et al. Gradual Rewarming with Gradual Increase in Pressure during Machine Perfusion after Cold Static Preservation Reduces Kidney Ischemia Reperfusion Injury. *PloS One* (2015) 10 (12):e0143859. doi: 10.1371/journal.pone.0143859

19. Yuzefovych Y, Valdivia E, Rong S, Hack F, Rother T, Schmitz J, et al. Genetic Engineering of the Kidney to Permanently Silence MHC Transcripts During ex vivo Organ Perfusion. *Front Immunol* (2020) 11:265. doi: 10.3389/fimmu.2020.00265

20. Al-Lamki RS, Bradley JR, Pober JS. Endothelial cells in allograft rejection. *Transplantation* (2008) 86(10):1340–8. doi: 10.1097/TP.0b013e3181891d8b

21. Lash LH. *In vitro* methods of assessing renal damage. *Toxicol Pathol* (1998) 26 (1):33–42. doi: 10.1177/019262339802600105

22. Nielsen PM, Laustsen C, Bertelsen LB, Qi H, Mikkelsen E, Kristensen ML, et al. *In situ* lactate dehydrogenase activity: a novel renal cortical imaging biomarker of tubular injury? *Am J Physiol Renal Physiol* (2017) 312(3):F465–f73. doi: 10.1152/ ajprenal.00561.2015

23. Yang WL, Ma G, Zhou M, Aziz M, Yen HT, Marvropoulos SA, et al. Combined administration of human ghrelin and human growth hormone attenuates organ injury and improves survival in aged septic rats. *Mol Med* (2016) 22:124–35. doi: 10.2119/ molmed.2015.00255

24. Sun X, Zhang C, Jin H, Sun G, Tian Y, Shi W, et al. Flow cytometric analysis of T lymphocyte proliferation in *vivo* by EdU incorporation. *Int Immunopharmacol* (2016) 41:56–65. doi: 10.1016/j.intimp.2016.10.019

25. Schett G, Elewaut D, McInnes IB, Dayer JM, Neurath MF. How cytokine networks fuel inflammation: Toward a cytokine-based disease taxonomy. *Nat Med* (2013) 19(7):822–4. doi: 10.1038/nm.3260

26. Siu JHY, Surendrakumar V, Richards JA, Pettigrew GJ. T cell allorecognition pathways in solid organ transplantation. *Front Immunol* (2018) 9:2548. doi: 10.3389/ fimmu.2018.02548

27. Vadori M, Cozzi E. The immunological barriers to xenotransplantation. *Tissue Antigens* (2015) 86(4):239–53. doi: 10.1111/tan.12669

28. Stewart ZA. Xenotransplantation: the contribution of CRISPR/cas9 gene editing technology. *Curr Transplant Rep* (2022) 9(4):268–75. doi: 10.1007/s40472-022-00380-3

29. Montgomery RA, Stern JM, Lonze BE, Tatapudi VS, Mangiola M, Wu M, et al. Results of two cases of pig-to-human kidney xenotransplantation. *N Engl J Med* (2022) 386(20):1889–98. doi: 10.1056/NEJMoa2120238

30. Wang W, He W, Ruan Y, Geng Q. First pig-to-human heart transplantation. *Innovation (Camb)* (2022) 3(2):100223. doi: 10.1016/j.xinn.2022.100223

31. Cowan PJ, Cooper DK, d'Apice AJ. Kidney xenotransplantation. *Kidney Int* (2014) 85(2):265–75. doi: 10.1038/ki.2013.381

32. Hamar M, Selzner M. Ex-vivo machine perfusion for kidney preservation. *Curr Opin Organ Transplant* (2018) 23(3):369–74. doi: 10.1097/MOT.0000000000000524

33. Rother T, Horgby C, Schmalkuche K, Burgmann JM, Nocke F, Jägers J, et al. Oxygen carriers affect kidney immunogenicity during ex-vivo machine perfusion. *Front Transplant* (2023) 2. doi: 10.3389/frtra.2023.1183908

34. Hamelink TL, Ogurlu B, De Beule J, Lantinga VA, Pool MBF, Venema LH, et al. Renal normothermic machine perfusion: the road toward clinical implementation of a promising pretransplant organ assessment tool. *Transplantation* (2022) 106(2):268–79. doi: 10.1097/TP.0000000000003817

35. Tomasi R, Tariq M, Hübner M, Strauss G, Längin M, Zeuzem-Lampert C, et al. T-cell response in a cardiac xenotransplant model. *Exp Clin Transplant* (2021) 19 (7):708–16. doi: 10.6002/ect.2020.0359

36. Ahrens HE, Petersen B, Ramackers W, Petkov S, Herrmann D, Hauschild-Quintern J, et al. Kidneys from α1,3-galactosyltransferase knockout/human heme oxygenase-1/human A20 transgenic pigs are protected from rejection during ex vivo perfusion with human blood. *Transplant Direct* (2015) 1(6):e23. doi: 10.1097/TXD.0000000000000533

37. Khalfoun B, Barrat D, Watier H, Machet MC, Arbeille-Brassart B, Riess JG, et al. Development of an ex vivo model of pig kidney perfused with human lymphocytes. Analysis of xenogeneic cellular reactions. *Surgery* (2000) 128(3):447–57. doi: 10.1067/msy.2000.107063

38. Piotti G, Palmisano A, Maggiore U, Buzio C. Vascular endothelium as a target of immune response in renal transplant rejection. *Front Immunol* (2014) 5:505. doi: 10.3389/fimmu.2014.00505

39. Eleftheriadis T, Pissas G, Crespo M, Nikolaou E, Liakopoulos V, Stefanidis I. A role for human renal tubular epithelial cells in direct allo-recognition by CD4+ T-cells and the effect of ischemia-reperfusion. *Int J Mol Sci* (2021) 22(4):1733. doi: 10.3390/ijms22041733

40. Schmalkuche K, Schwinzer R, Wenzel N, Valdivia E, Petersen B, Blasczyk R, et al. Downregulation of swine leukocyte antigen expression decreases the strength of xenogeneic immune responses towards renal proximal tubular epithelial cells. *Int J Mol Sci* (2023) 24(16):12711. doi: 10.3390/ijms241612711

41. Plege-Fleck A, Lieke T, Römermann D, Düvel H, Hundrieser J, Buermann A, et al. Pig to rat cell transplantation: reduced cellular and antibody responses to xenografts overexpressing PD-L1. *Xenotransplantation* (2014) 21(6):533–42. doi: 10.1111/xen.12121

42. You K, Gu H, Yuan Z, Xu X. Tumor necrosis factor alpha signaling and organogenesis. *Front Cell Dev Biol* (2021) 9:727075. doi: 10.3389/fcell.2021.727075

43. Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. *Front Immunol* (2014) 22(5):491. doi: 10.3389/fimmu.2014.00491

44. Jang DI, Lee AH, Shin HY, Song HR, Park JH, Kang TB, et al. The role of tumor necrosis factor alpha (TNF-α) in autoimmune disease and current TNF-α Inhibitors in therapeutics. *Int J Mol Sci* (2021) 22(5):2719. doi: 10.3390/ijms22052719

45. Mehta AK, Gracias DT, Croft M. TNF activity and T cells. *Cytokine* (2018) 101:14–8. doi: 10.1016/j.cyto.2016.08.003

46. Choy JC. Granzymes and perforin in solid organ transplant rejection. *Cell Death Differ* (2010) 17(4):567–76. doi: 10.1038/cdd.2009.161

47. Kummer JA, Wever PC, Kamp AM, ten Berge IJ, Hack CE, Weening JJ. Expression of granzyme A and B proteins by cytotoxic lymphocytes involved in acute renal allograft rejection. *Kidney Int* (1995) 47(1):70–7. doi: 10.1038/ki.1995.8

48. Plege A, Borns K, Beer L, Baars W, Klempnauer J, Schwinzer R. Downregulation of cytolytic activity of human effector cells by transgenic expression of human PD-ligand-1 on porcine target cells. *Transpl Int* (2010) 23(12):1293–300. doi: 10.1111/j.1432-2277.2010.01130.x

49. Le Bas-Bernardet S, Blancho G. Current cellular immunological hurdles in pig-to-primate xenotransplantation. *Transpl Immunol* (2009) 21(2):60–4. doi: 10.1016/j.trim.2008.10.006

50. Halawi A, El Kurdi AB, Vernon KA, Solhjou Z, Choi JY, Saad AJ, et al. Uncovering a novel role of focal adhesion and interferon-gamma in cellular rejection of kidney allografts at single cell resolution. *Front Immunol* (2023) 14:1139358. doi: 10.3389/fimmu.2023.1139358

51. Zhang G, Iwase H, Li Q, Yamamoto T, Jagdale A, Ezzelarab MB, et al. The role of interleukin-6 (IL-6) in the systemic inflammatory response in xenograft recipients and in pig kidney xenograft failure. *Front Immunol* (2021) 12:788949. doi: 10.3389/fimmu.2021.788949

52. Batal I, De Serres SA, Mfarrej BG, Grafals M, Pinkus GS, Kalra A, et al. Glomerular inflammation correlates with endothelial injury and with IL-6 and IL-1β secretion in the peripheral blood. *Transplantation* (2014) 97(10):1034–42. doi: 10.1097/01.TP.0000441096.22471.36

53. Karczewski M, Karczewski J, Poniedzialek B, Wiktorowicz K, Glyda M. Cytometric analysis of TH1/TH2 cytokines in the urine of patients undergoing kidney transplantation. *Ann Transplant* (2009) 14(3):25–8.

54. Mota AP, Vilaça SS, das Mercês FL Jr., Pinheiro Mde B, Teixeira-Carvalho A, Silveira AC, et al. Cytokines signatures in short and long-term stable renal transplanted patients. *Cytokine* (2013) 62(2):302–9. doi: 10.1016/j.cyto.2013.03.001

55. Juneja VR, McGuire KA, Manguso RT, LaFleur MW, Collins N, Haining WN, et al. PD-L1 on tumor cells is sufficient for immune evasion in immunogenic tumors and inhibits CD8 T cell cytotoxicity. *J Exp Med* (2017) 214(4):895–904. doi: 10.1084/jem.20160801

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 07 February 2024
DOI 10.3389/fimmu.2024.1355609

OPEN ACCESS

EDITED BY
Guido Moll,
CharitéUniversity Medicine Berlin,
Germany

REVIEWED BY
Eckhard Wolf,
Ludwig Maximilian University of Munich,
Germany
Rafael Miyashiro,
University of Miami, United States

*CORRESPONDENCE
Wayne J. Hawthorne
Wayne.hawthorne@sydney.edu.au

RECEIVED 14 December 2023
ACCEPTED 22 January 2024
PUBLISHED 07 February 2024

CITATION
Hawthorne WJ (2024) Ethical and legislative advances in xenotransplantation for clinical translation: focusing on cardiac, kidney and islet cell xenotransplantation.
Front. Immunol. 15:1355609.
doi: 10.3389/fimmu.2024.1355609

Ethical and legislative advances in xenotransplantation for clinical translation: focusing on cardiac, kidney and islet cell xenotransplantation

Wayne J. Hawthorne[1,2]*

[1]The Centre for Transplant & Renal Research, Westmead Institute for Medical Research, Westmead, NSW, Australia, [2]Department of Surgery, School of Medical Sciences, University of Sydney, Westmead Hospital, Westmead, NSW, Australia

In this state-of-the-art review we detail the journey of xenotransplantation from its infancy, detailing one of the first published cases and the subsequent journey the field took in its inception and development. With a focus on the science, technological advances, precautions required along with the potential limitations in application, the ethics, guidance's, and legislative advances that are required to reach the safe and efficacious clinical application of xenotransplantation. Along with a view over the past several decades with the overall significant advancements in pre-clinical study outcomes particularly in islet, kidney, and heart xenotransplantation, to ultimately reach the pinnacle of successful clinical heart and kidney xenotransplants. It outlines the importance for the appropriate guidance's required to have been developed by experts, scientists, clinicians, and other players who helped develop the field over the past decades. It also touches upon patient advocacy along with perspectives and expectations of patients, along with public opinion and media influence on the understanding and perception of xenotransplantation. It discusses the legislative environment in different jurisdictions which are reviewed in line with current clinical practices. All of which are ultimately based upon the guidance's developed from a strong long-term collaboration between the International Xenotransplantation Association, the World Health Organisation and The Transplantation Society; each having constantly undertaken consultation and outreach to help develop best practice for clinical xenotransplantation application. These clearly helped forge the legislative frameworks required along with harmonization and standardization of regulations which are detailed here. Also, in relation to the significant advances in the context of initial xeno-kidney trials and the even greater potential for clinical xeno-islet trials to commence we discuss the significant advantages of xenotransplantation and the ultimate benefit to our patients.

KEYWORDS

ethics, guidance's, hyperacute rejection, legislation, xenozoonosis, xenotransplantation

1 Introduction

Xenotransplantation, the latest frontier in transplantation is the process of retrieving organs, tissues or cells from one species and transplanting them into another. It has long been heralded as the ultimate solution to the overwhelming shortage of human organs available for transplantation (1). The concept of utilizing non-human organ and tissue sources to meet the overwhelming demand on conventional donors has captured the attention of clinicians, scientists, healthcare providers, and patients alike for many decades but has also been a concept for hundreds of years (Figure 1). As can be seen in Figure 1, which is a timeline of some of the major landmarks in the journey of xenotransplantation. The first published attempts of xenotransplantation occurred with xeno-transfusion occurring in the 1600's then in the 1800's xeno-skin transplants were attempted prior to more ambitious attempts at kidney xenotransplantation. There has been a long line of endeavor as advancements in medical science and technology have brought the prospect of xenotransplantation closer to reality. Importantly the ethical and legislative landscape surrounding this pioneering field has undertaken renewed and ever-increasing attention but still requires ongoing updates (2–4). A large effort from the International Xenotransplantation Association (IXA) in conjunction with others such as the World Health Organisation (WHO) and the Transplantation Society (TTS) have been constantly undertaken, however as the field progresses more needs to be done from a broader international and national regulatory perspective.

Xenotransplantation offers us the potential to save countless lives by providing a readily available supply of organs, tissues and cells, significantly reducing the waiting time for transplants, and alleviating the suffering of patients on transplant wait lists. It is also a major means by which we can actively abolish the trade in trafficked organs and organ transplant tourism. However, with this promise comes a complex web of ethical considerations and legal frameworks that must be carefully navigated to ensure the responsible and ethical translation of xenotransplantation from the laboratory to the clinic. With this we must ensure that the same endemic issues do not occur with xenotransplantation that have occurred with human organ transplantation such as xenotransplant tourism (5) and unethical processes used to make profits at the expense of the animals used and the patients that may be misled into undertaking unapproved procedures (6).

Historically, xenotransplantation has faced significant challenges, including the perceived/potential for the transmission of diseases from animals to humans (xenozoonosis) (7), concerns over animal welfare (when breeding and producing the donor animals) (8), cultural and religious issues particularly the notion of crossing species boundaries in the use of their tissues for transplantation (6). These challenges led to the imposition of strict regulations along with embargos and a nuanced ethical debate that continues to shape the direction of the field. In recent years, ground-breaking advancements in genetic engineering has rapidly accelerated the field which offers new hope, massively advancing the creation of genetically modified pigs with organs engineered for compatibility with the human immune system (9). These developments have paved the way for the initiation of trials in humans involving xeno-hearts approved under "compassionate use" for live patients (10) along with xeno-hearts and kidneys being studied clinically in a new model using "Brain Dead" recipients (11, 12) along with very successful preclinical trials using transgenic pig islet cells (13).

In this review, we explore the ethical and legislative advances that are underpinning xenotransplantation as it moves toward broadly accepted clinical translation. We delve into the ethical considerations surrounding xenotransplantation, examining questions related to the potential risks of xenozoonotic disease transmission, animal rights, their use, and the public's perception of this innovative medical approach. We also survey the changes in legislative frameworks governing xenotransplantation, charting their evolution over time, and highlighting the necessity of harmonization and standardisation in regulations worldwide. With a focus on what has been undertaken from the peak governing bodies of the WHO, TTS and the IXA (4)

As the initial clinical trials of xeno-kidneys (12) and hearts (14) bring us closer to the long-awaited reality of xenotransplantation, it is imperative to reflect on the ethical and legislative progress that has brought us to this pivotal moment (4). The careful balance between scientific innovation, human health, and ethical responsibility is at the heart of this transformational journey, and it is through a comprehensive understanding of these advances that we can move forward confidently, ethically and legislatively with the world focusing on xenotransplantation (6).

2 Historical perspective

Xenotransplantation has long been heralded as a potential solution to the overwhelming shortage of human organs, tissues and cells available for transplantation (1). The concept of utilizing non-human sources to meet the organ demand has captured the imagination of scientists, healthcare providers, and patients alike. As advancements in science and technology have brought the prospect of xenotransplantation closer to reality, the ethical and legislative landscape surrounding this pioneering field has gained increasing attention especially with the last few years of accelerated progress and commencement of limited life-saving heart and kidney xenotransplantation which have been approved under special compassionate use authorization (i.e., a specific treatment for patients with immediate life-threatening conditions to have access to investigational products outside of an U.S Food and Drug Administration (FDA) -approved clinical trial when no comparable or alternative therapeutic treatment exists to treat the patient's life threatening illness) (10–12, 15).

Historically, xenotransplantation's journey has been marked by both hope and challenge. As seen in Figure 1. Xenotransplantation has

Abbreviations: αGal, galactose-α1,3-galactose; BD, brain-dead; DPF, designated pathogen free; IXA, International Xenotransplantation Association; NHP, non-human-primates; PERV, porcine endogenous retroviruses; TTS, the Transplantation Society; FDA, Food and Drug Administration; WHO, World Health Organisation; WT, wild-type.

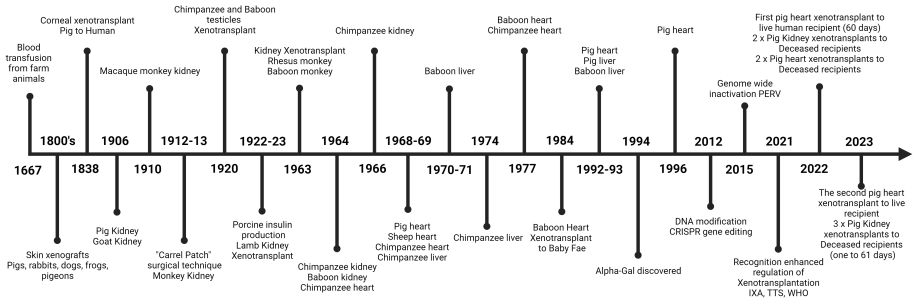


FIGURE 1
A concise timeline of some of the major developments that have occurred in the field of xenotransplantation. From the first recorded attempt of a xeno-blood transfusion to the current advent of successful clinical xenotransplants of transgenic pig organs to humans.

been attempted in many and various settings with many unusual attempts from rather bizarre initial concepts and treatments to now becoming clinical reality. The first published attempt of xenotransplantation took place in the early 17th century when xeno-transfusion was first attempted in June of 1667, in Paris. Jean-Baptiste Denis, a French physician, doctor of King Louis XIV, and Paul Emmerez, surgeon, transfused what we assume to be a small amount of blood from a lamb into a 15-yr-old boy (16). Unfortunately, on the second attempted use of xeno-transfusion it proved unsuccessful and resulted in the death of the patient after which xeno-transfusion was outlawed by the French government (16).

In 1906 the first reported successful kidney xenotransplant was carried out by Mathieu Jaboulay after he and Alexis Carrel perfected the technique of vascular anastomosis. Jaboulay used the vascular technique to transplant a pig kidney onto the brachial artery and cephalic vein of a 48-yr-old woman. Immediately and for the first day and a half he saw significant urine output, but on the third day, he was forced to remove the kidney because of vascular thrombosis (17). Sadly, a lack of understanding of immunology, hematology and any of the intricacies of transplantation, let alone the issues of cross-species xenotransplantation prevented any chance of longer-term success. These early attempts were characterized by a lack of ethical and scientific groundwork, and the risks and consequences of such procedures were often not well understood.

The ensuing centuries saw sporadic and largely unsuccessful attempts at xenotransplantation, with frequent instances of graft rejection and infections that further tempered enthusiasm for the field (18–20). Moreover, as medical ethics evolved and animal welfare concerns gained prominence, the scientific community was challenged to grapple with the significant ethical implications of these procedures, especially when it came to the use of animals involved in the pre-clinical trials and as a source of organs, tissues and cells for transplantation into humans (6).

In the latter half of the 20th century, with the advent of organ transplantation and the increasing demand for donor organs, the potential of xenotransplantation was revisited with renewed enthusiasm pushing the field forward. The discovery of alpha Gal as the mechanism responsible for causing hyperacute rejection (HAR) (21) and the concept of utilizing specifically designed genetically modified pigs (9, 13, 22), capable of providing organs less immunogenic to the human immune system, marked a significant turning point in xenotransplantation's history. These developments paved the way for the initiation of initial clinical trials involving xeno-kidneys and soon to be islet cell xenotransplants.

As we explore the ethical and legislative advances propelling xenotransplantation toward clinical translation, we must acknowledge the lessons of history. The historical backdrop of early, less-informed attempts, coupled with ethical concerns, has played an instrumental role in shaping the ethical and legislative frameworks we see today (23). The careful balance between scientific innovation, human health, and ethical responsibility is at the heart of this transformational journey. It is through an understanding of these historical challenges that we can appreciate the significance of the ethical and legislative advances discussed in this review, as they propel us closer to the long-awaited reality of xenotransplantation that now seems to be underway (11, 14).

3 Major ethical considerations

The remarkable potential of xenotransplantation to address the critical shortage of human organs has been met with considerable ethical scrutiny, raising profound questions and dilemmas that must be thoughtfully addressed. A number of the core areas of ethical concern that have been central to the discourse surrounding xenotransplantation are: the potential for xenozoonosis, public and regulatory issues, crossing of species boundaries and ensuring appropriate animal ethics. However, these must be balanced against the absolute positive gains for the overwhelming number of potential patients that can benefit from xenotransplantation when there are so many medical, financial and social issues for these patients. As can be seen in Figure 2, the balance between the negative aspects of their disease versus receiving a cure from the transplant is overwhelmingly weighted to the positive. This is because the benefits far outweigh the problems of ongoing and increasing ill health, secondary complications, invalidity and ultimately death. However, there are not enough human donor organs available for transplantation and using this single example, the case of patients suffering from type 1 diabetes, there are innumerable patients that could benefit from islet cell xenotransplantation with it being life changing and lifesaving.

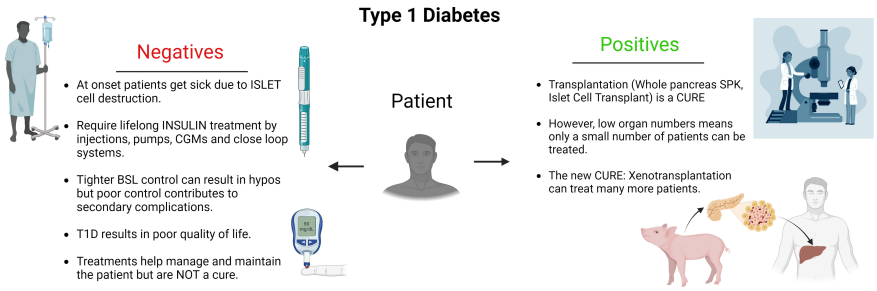


FIGURE 2
Diagram detailing the conundrum faced by patients suffering from Type 1 diabetes. From the impactful, negative factors affecting them resulting from their disease. To the positive outcomes achieved by having a transplant and the capacity for xenotransplantation to offer more patients a cure.

3.1 Potential xenozoonosis

One ethical concern intrinsic to xenotransplantation relates to the potential for the transmission of diseases from animals to humans, a phenomenon known as xenozoonosis. The concept of transmission although theoretical is not unfounded, as various pathogens, including retroviruses, have been identified in pigs could be potential threats in immunocompromised transplant recipients and then theoretically spread to direct close contacts and the broader community (24, 25).

As such this raises potential ethical dilemmas. The duty to protect the broader community and prevent the spread of theoretically potential infectious diseases must be weighed against the need to explore novel medical solutions to help these patients suffering from end stage organ failure and other diseases (6). The possibility of creating animals free from such pathogens as porcine endogenous retroviruses (PERV) through genetic engineering (26) has already been shown to be possible along with raising donor animals in designated pathogen free (DPF) facilities. Along with pigs that have limited pathogens including restricted PERV (27) or where studies have shown no potential for transmission (28, 29). Despite best intensions and even following screening of donor animals we have seen that donor pig organs can still potentially have undetectable porcine viruses such as cytomegalovirus or porcine roseolovirus (PCMV/PRV) detected posttransplant in the donor tissue by plasma microbial cell-free DNA (30). This occurred despite pre-transplant screening and following transplantation into a patient (25).

From a patient and community perspective it is therefore essential for the patients, their family and immediate direct contacts along with the community to understand that if there were in fact a positive case of transmission of a xenozoonosis into a xenotransplant patient that there may well be serious implications to all involved. These implications are potentially as severe as life-long restriction and quarantine of the recipient and may extend to their direct close contacts (25). As part of all Xenotransplantation trial participation it has been advised by the WHO and IXA "Changsha Communique" that all xenograft recipients' commit to lifelong xenozoonotic monitoring, including agreement to quarantine as a measure to prevent any serious potential spread of infection if detected or suspected (4, 31). As part of the enlistment and education process of patients, patients should be advised of these requirements at the time of prospective trial participation and informed consent process. If the participants choose to they should have the right to withdraw from a xenotransplant trial prior to transplantation. However, once they have been informed, consented and commenced in the trial having undergone xenotransplantation, recipients would be subject to the regulations governing infection containment at a National and International level. Most countries have in place legislation that enforces such quarantinable regulations in relation to communicable diseases (8, 32, 33).

As additional safeguards we also have significant arrays of new antiviral agents capable of eliminating or treating such disease potential (34). Yet, it is essential to ensure that the risk of transmission is minimized and that robust safety measures are in place to protect recipients. This however, does require further address by responsible organizations (WHO, TTS, IXA) and legislators in the many and various international jurisdictions (2–4).

3.2 Public and regulatory support

Addressing these ethical concerns is not only a moral imperative but also crucial for gaining public and regulatory support for xenotransplantation. Public perception of the ethics surrounding xenotransplantation can significantly influence its acceptance and, consequently, the regulatory environment. As such there has been significant engagement with both societal and religious organizations to ensure robust understanding of the key concepts and garner opinion and support (6, 35, 36).

The IXA has endeavored to undertake public engagement with ongoing dialogue which are essential to fostering trust with transparency and acceptance. It is incumbent on the broader scientific community and policymakers to communicate the steps taken to mitigate ethical concerns and to provide evidence of the rigorous ethical oversight and animal welfare standards applied in xenotransplantation research. Furthermore the governing organization of xenotransplantation the IXA is maintaining its mission to promote xenotransplantation as a safe, ethical, and effective therapeutic modality by; fostering the science of xenotransplantation through promotion of ethical clinical and pre-clinical research, productive discourse, and collaboration;

along with further educating health care providers and lay persons through broad, representative participation in interactive public debate; and also guiding the development of scientifically sound, internationally consistent public policy that is responsive to new developments in the field and acknowledges varying social, ethical and legal frameworks (37). Along with ongoing engagement with regulatory bodies and other agencies to ensure they balance the advancement of science but safeguarding the ethical principles. This is actively being undertaken with a strong push from the IXA to engage numerous agencies globally to ensure this continues to occur and keep pace with the rapidly developing technologies (8).

3.3 Crossing species boundaries

Xenotransplantation challenges the traditional conceptual boundaries that separate humans from animals. It poses profound philosophical and ethical questions about the nature of different species and the moral obligation we owe to different species. As we engage in practices that involve genetic modification and the use of animals for human benefit, the ethical boundaries are changing with increasing pressure on ethics committees and legislators to keep track with the pace of change, and we have a moral imperative to ensure that we do keep pace and provide adequate oversight (6).

Some ethicists argue that xenotransplantation exemplifies the Anthropocentric approach (38), emphasizing human interests over those of animals, while others advocate for a more inclusive biocentric perspective that values all forms of life equally (39). The challenge is to find a balance between medical innovation and ethical responsibility to both animal and man (6).

3.4 Animal welfare

Xenotransplantation necessitates the use of animals as organ donors. Pigs being primarily used due to their physiological compatibility with humans, their ability to be bred in large numbers at a rapid rate, and their ability to be readily genetically altered. This along with their longstanding acceptance as a source of medical products such as drugs and heart valves and other decellularized tissues. With by far the strongest reason being there acceptance as a major food supply and source of products for man for as long as they have been domesticated should ensure their ease of use ethically (6). However, the welfare of these animals is of paramount concern. As some organizations push the principal that pigs are not merely commodities but sentient beings with the capacity to experience pain and suffering.

The major issues raised are to ensure the donor pigs are being ethically and humanely cared for and ensure the process of genetic manipulation does not cause them any health issues. One could argue that the facilities and conditions that these animals are housed and the care they receive is of a superior level to a normal commercial piggery due to the highly controlled and run facilities including the need for donor animals to be in DPF facilities. Underpinning this is the fact that all animal research projects including the breeding of, care and handling of the animals are undertaken in strict compliance under animal ethics legislation and under scrutiny of ethics committees (6). The genetic modifications required for these donor animals have been carefully designed to ensure they do not affect the health of the source pigs at all. Therefor from an ethical standpoint the level of suffering could be perceived as minimal or negligible. On the other hand, the potential benefit for patients is very high, being lifesaving and life changing (6).

There are also the ethical concerns for pre-clinical study recipients the various animals used and especially the non-human-primates (NHP) which are the benchmark for preclinical trials. Their use is highly recommended prior to acceptance of any program moving to the clinic, and has been advised in many guidance's such as the "Changsha Communique" that recommend their use to provide safe and efficacious treatment regimen and modalities prior to commencement of any clinical trials (4). So much so that the United States, Food and Drug Administration (FDA) reviewed the NHP preclinical data prior to granting permission for the Baltimore, MD, USA XenoHeart team at the University of Maryland School of Medicine approval for the first pig-to-human heart transplant to be granted (40). The strictest of compliance on ethical grounds is required for any animal study let alone the massive scrutiny undertaken by authorities for NHP research related projects. In most jurisdictions special permission is required, even following appropriate animal ethics approval. Researchers are only allowed to undertake any study with NHPs once accredited and specifically approved due to community concern for their care as they are viewed as so similar to humans.

Scholars and ethicists have explored various strategies to mitigate these concerns. The concept of "minimum moral standing," as proposed by Rollin, asserts that pigs raised for xenotransplantation should be provided with living conditions and treatment that accord them a minimum level of moral consideration (41). This includes efforts to reduce suffering and enhance the overall welfare of the animals. Ethical guidelines and regulations often inspired by principles of animal welfare, have been developed to ensure humane treatment throughout the animals' lives and the very best moral and ethical care for all animals.

4 Legislative frameworks

Xenotransplantation stands at the intersection of cutting-edge medical science and a complex regulatory landscape. The ethical and safety concerns surrounding xenotransplantation have led to the development of a multifaceted legislative framework designed to ensure both the advancement of this field and the protection of public health.

4.1 Existing legislative frameworks

Legislative oversight of xenotransplantation varies across different countries and regions. In the United States (USA), the Food and Drug Administration (FDA) and the Centres for Medicare and Medicaid Services (CMS) (8, 42) are the primary regulatory bodies tasked with overseeing xenotransplantation research and clinical trials. While in China it is the Chinese FDA,

Korea (Korean FDA), Argentina (AFDA) whilst in Europe it is the European Medicines Agency (EMA) (43) and in Australia the Therapeutics Goods Administration (TGA) that are charged with establishing regulations and underpinning legislation to support this.

These existing frameworks typically encompass some updates to their regulations, including safety assessments, informed consent, monitoring for xenozoonotic diseases, and research and clinical trial oversight. Such legislation should ideally aim to strike a balance between encouraging scientific innovation and ensuring that risks are rigorously assessed and mitigated with a background based upon preclinical trials, some requiring or suggestive of non-human primate trials as a lead-in to proof of concept for clinical trials (4, 8, 42).

4.2 Evolution of legislative frameworks

The legislative landscape for xenotransplantation has evolved significantly over the years. As science has advanced, the regulations have been adapted to keep pace with the changing landscape in transplantation but it still lacks the oversight and ability to completely control all that occurs. Despite the best policies and guidance's more universal legislation is required to outlaw and prevent organ trafficking and ongoing issues associated with unscrupulous operators (44). The early years of xenotransplantation were characterized by limited regulatory oversight and fragmented approaches to the management of potential risks due to limited legislation to this new field. However, significant oversight was established early on by the WHO, TTS and IXA to ensure there were guidance's developed to underpin the field (2–4).

However, high-profile setbacks and scientific developments have prompted a revaluation of regulatory frameworks. An example of this was the identification of porcine endogenous retroviruses (PERVs) which raised concerns by government legislators about the potential transmission of these retroviruses to immunocompromised transplant recipients (45). As a result, several jurisdictions-initiated moratorium preventing any clinical xenotransplantation trials from commencing and as such a stronger focus was placed on the assessment and management of this risk in regulatory guidelines (46).

Recently, regulatory bodies have intensified their efforts to provide comprehensive guidelines for xenotransplantation, reflecting a growing recognition of the field's incredible potential with a balance against the risks. Some guidance's such as from the USA FDA have addressed issues such as genetic modifications in source animals, monitoring for infectious agents, and the ethical treatment of donor animals (42). And the USA government and other jurisdictions legislating and licensing biological products such as xenografts, tissues and cells under specific biological products legislation (47).

4.3 Harmonization and standardization of regulations

One of the most pressing needs in the field of xenotransplantation is the harmonization and standardization of regulations on an international basis. Currently, different countries jurisdictions have distinct legislative frameworks, which can create challenges for researchers and clinicians working in the field. These disparities can hinder the progress of clinical trials and create unnecessary hurdles for advancing this promising technology. Harmonization and standardization are essential for streamlining the path from research to clinical application. By establishing consistent regulations that are internationally recognized and harmonized, xenotransplantation can transcend geographical boundaries, allowing for more efficient and effective collaboration among researchers and acceptance of international clinical trials and also their results (48). As such the IXA in conjunction with the TTS and WHO have for the past decades have been undertaking significant engagement since they combined efforts to establish guidance's and a xenotransplant registry (49). A significant amount of work has been done by these organizations to ensure there has been expert consultation at an international level. A number of high-level consultations have resulted in the design and development of internationally established guidance's published under the IXA, TTS, and WHO frameworks with the first published in 2008 with the "Changsha Communique" being drafted and guidance's now update by multiple panels of international experts on multiple occasions (2–4).

5 Clinical xenotransplant studies

The transition from laboratory research to clinical practice is a pivotal phase in the journey of xenotransplantation, and it is marked by the initiation of clinical studies of various kind. These have to date involved the transplantation of organs or tissues from genetically modified pigs into human recipients. In recent years, two types of clinical studies have gained prominence: xeno-heart and kidney transplantation (50–52).

5.1 Overview of initial xeno-cardiac, kidney and islet cell trials

5.1.1 Xeno-cardiac and kidney clinical programs

Current clinical studies involving xeno-heart and kidney transplantation have sought to address the critical shortage of available human organs for transplantation. These studies have used specifically developed transgenic pigs that have been genetically modified to be less immunogenic, coagulopathic and prevent hyperacute xenograft rejection.

To date two successful long-term transgenic pig heart xenotransplants into live human patients have been undertaken (40). They have been defined as successful on multiple levels. Firstly, and most importantly they did not undergo hyperacute xenograft rejection, the primary and most significant barrier to xenograft success. Secondly, on the ground of function, these hearts were functional and life supporting for several months. Lastly, the patients were off VA-ECMO, extubated and on no supportive inotropic agents with normal cardiac index and normal biventricular function as demonstrated by echocardiography (14). It is important to understand that both pig-to-human heart

xenotransplants were performed following permission for the procedures being granted under Expanded Access authorization by the United Stated, FDA (also known as "compassionate use") (40).

There have also been several transgenic pig kidney and heart xenotransplants performed in a new clinical recipient research modality. These few early attempts have utilized brain-dead (BD) recipients for transplantation studies and are in their very early stages, having faced various challenges from an ethical perspective. These studies have once again utilized transgenic pigs as the source of donor organs. They have been specifically produced to avoid hyperacute xenograft rejection and provide function in human patients. The first of these kidney studies were taken to only 74 hours posttransplant due to strict ethical constraints. Despite this no hyperacute rejection was observed, and the kidneys remained viable until termination with no chimerism or transmission of porcine retroviruses detected (11). There were two transgenic pig heart xenotransplants also performed in two recently deceased BD recipients. These were only able to be run to 66 hours posttransplant again due to ethical constraints of this model. For both hearts, they also found no evidence of cellular or antibody-mediated rejection, as assessed using histology, flow cytometry and a cytotoxic crossmatch assay. Moreover, they found no evidence of zoonotic transmission from the donor pigs to the human recipients (52).

The transgenic pig kidney xenotransplant studies have continued with several others being undertaken in the same modality in BD recipients. The most recent having been taken out as far as 61 days posttransplant. Despite favorable short-term outcomes and absence of hyperacute injuries, their findings suggest that antibody-mediated rejection in transgenic pig-to-human kidney xenografts might be occurring. The caveat here being the limited transgenesis of these particular donor pig organs playing a significant role (53).

Despite these initial issues and the question of validity of testing the xeno-kidneys in BD recipients due to their altered metabolic state, they represent a promising approach to expand the way to test the safety and efficacy of xeno-organs prior to undertaking xenotransplants in clinical trials. These studies have the potential to increase the data to support the use of xeno-organs to increase the pool of available organs for patients with end-stage renal disease (11, 54).

5.1.2 Islet cell trials

Islet cells, clusters of cells in the pancreas that produce insulin, have been the focus of many pre-clinical trials aiming to provide a treatment for type 1 diabetes. In these studies, islet cells from genetically engineered pigs have been transplanted into various animal models where they have had diabetes induced and are transplanted to potentially restore insulin production. For decades there have also been a significant number of early attempts with both free and encapsulated islets to treat human patients suffering from Type 1 diabetes (55, 56). These have had variable results and no study to date has shown significant change or complete resolution of the recipient's diabetic state. This has been due to the use in the most part of wild type pig islets rather than purpose developed and bred transgenic pigs (55, 56). However, results from preclinical xeno-islet trials have shown great promise in improving glucose control in non-human-primates establishing it as a potential therapeutic modality for treating diabetic patients (13).

5.2 Significance of clinical trials in advancing xenotransplantation

The significance of clinical xeno-heart, kidney and islet cell trials in moving xenotransplantation towards clinical reality cannot be overstated. These trials mark a crucial step in the validation of the safety and efficacy of xenotransplantation in humans. Their outcomes will inform researchers, healthcare providers, regulatory bodies, and the public about the feasibility of this innovative medical approach (57, 58).

Successful trials may also pave the way for wider acceptance of xenotransplantation as a viable solution to the organ shortage crisis. By demonstrating the effectiveness of modified pig organs and addressing safety concerns, clinical trials can build the case for regulatory approval and wider adoption (46, 59).

6 Patient perspectives

The success and acceptance of xenotransplantation hinge not only on scientific progress but also on the perspectives and expectations of patients who may ultimately benefit from this innovative medical approach. Understanding the views of prospective recipients and incorporating their voices is essential for the responsible advancement of xenotransplantation (60).

6.1 Perspectives and expectations of patients

Patients facing organ failure or debilitating medical conditions have high expectations for xenotransplantation. They see it as a beacon of hope, offering the prospect of a healthier and more fulfilling life. For patients on waiting lists for human organs, xenotransplantation represents a potential lifeline, providing the promise of shorter waiting times and increased access to transplantation.

However, it's crucial to recognize that patients also have concerns and uncertainties, including the long-term outcomes of xenotransplantation, potential health risks, and the implications of receiving an organ from another species. Patient perspectives encompass a range of emotions, from hope and optimism to apprehension and caution. Addressing these concerns and providing accurate information is paramount in ensuring patient engagement and consent (61).

6.2 Informed consent and patient advocacy

In the realm of clinical trials for xenotransplantation, informed consent is a cornerstone of ethical practice. Patients must be fully informed about the experimental nature of the procedure, the potential risks, and the expected benefits. Informed consent allows patients to make autonomous decisions and plays a vital role in respecting their autonomy (39).

Patient advocacy organizations and support networks also play a crucial role in ensuring that patient perspectives are heard and addressed. These organizations work to protect patients' rights, advocate for transparency, and provide a platform for patients to voice their concerns and expectations. Their role in the xenotransplantation landscape is pivotal in safeguarding the interests of patients.

Patient perspectives and informed consent are not only ethical imperatives but also contribute to the overall success and sustainability of xenotransplantation. By ensuring patients are well-informed and actively engaged in the decision-making process, the field can progress responsibly and ethically, addressing the hopes and concerns of those it aims to benefit (13).

7 Public opinion and media influence

Public opinion and media coverage play a pivotal role in shaping the trajectory of xenotransplantation, influencing public perception, regulatory decisions, and the overall direction of this ground-breaking field.

7.1 Shaping the future of xenotransplantation

Public opinion wields a considerable impact on the acceptance and progress of xenotransplantation. As a novel medical approach with ethical and scientific complexities, xenotransplantation has the potential to stir both excitement and apprehension among the public. Positive public sentiment can foster support for research, funding, and regulatory approvals, whereas negative perceptions may hinder its advancement (62).

Media coverage significantly influences public opinion by serving as a primary source of information and shaping public discourse. Journalistic narratives can frame xenotransplantation as a ground-breaking medical solution or alternatively in a negative way posing it as a scary and risky endeavor, impacting how it is perceived by the masses (63). It is therefore imperative that the media provides balanced, accurate, and accessible information and in doing so will be vital in shaping the future of xenotransplantation.

7.2 Disseminating information and potential misconceptions

Media outlets serve as conduits for disseminating information about xenotransplantation. The media plays an important role in educating the public about the science, ethics, and potential benefits of xenotransplantation. However, the media can also perpetuate misconceptions, oversimplify complex issues, or sensationalize scientific advancements, which may lead to unwarranted public fears and concerns.

The responsible dissemination of information is paramount. Accurate, balanced, and well-informed media coverage is essential in fostering a constructive public dialogue, minimizing misconceptions, and ensuring that public sentiment is based on sound knowledge. Scientists, healthcare providers, and the xenotransplantation community have a shared responsibility to engage with the media to provide accurate and clear information (63, 64).

Public opinion and media influence are pivotal factors in the development of xenotransplantation, influencing the degree of support, funding, and public acceptance. The media's role in accurately disseminating information and minimizing misconceptions is key to ensuring that public opinion is well-informed and that decisions regarding the future of xenotransplantation are made based on a balanced understanding of the risks and benefits (65).

8 International collaboration for xenotransplantation

International collaboration is a cornerstone of xenotransplantation research, and its significance extends to the establishment of common standards, guidelines, and best practices. This global cooperation is crucial for the responsible advancement of the field and the harmonization of regulatory and ethical frameworks.

8.1 Importance of global collaboration

Xenotransplantation is not limited by geographic boundaries as seen in the geographical makeup of the broad membership of the IXA and of the significant publications from various units around the world. Researchers, scientists, and healthcare providers contribute their expertise and insights to propel this innovative field forward and the pre-clinical and novel and new use of models such as the BD recipient are synergistic and provide novel information that is perceived to not able to be achieved in NHP. The sharing of knowledge, data, and research findings fosters a collective understanding of the complexities involved in xenotransplantation (66).

Global collaboration is essential in harnessing diverse perspectives and experiences to address common challenges, such as the prevention of zoonotic diseases, the ethical treatment of animals, and the assessment of safety and efficacy (67, 68). This collective effort accelerates the translation of xenotransplantation from research to clinical practice and ensures that there is minimal risk of xenozoonosis or other potential issues (48).

8.2 Establishing common standards and guidelines

International collaboration in xenotransplantation research also enables the establishment of common standards, guidelines, and best practices. As the field progresses, consensus on regulatory, ethical, and scientific parameters becomes increasingly vital. Such harmonization streamlines the path from research to clinical application.

Common standards ensure that xenotransplantation research adheres to shared principles, such as animal welfare, patient safety, and ethical practices. International cooperation allows for the identification of gaps and discrepancies in current regulatory frameworks, enabling the development of more comprehensive and universally applicable guidelines such as the "Changsha Communique" (4).

Global collaboration in xenotransplantation research is not merely a choice but a necessity. By pooling resources, knowledge, and expertise from diverse regions, the field can progress with a unified vision. International cooperation helps establish common standards and guidelines, facilitating the responsible and ethical advancement of xenotransplantation and its translation to clinical reality (31) along with ensuring the registries are supported to be able to capture and report on the fields clinical efforts (69).

9 Conclusion

The journey of xenotransplantation, the transplantation of organs or tissues from one species to another, has witnessed significant advancements and encountered ethical, legislative, and scientific challenges. This review has delved into various facets of xenotransplantation, emphasizing its potential to address the critical organ shortage crisis while highlighting the essential elements required for its responsible and successful translation to clinical reality.

9.1 Take home messages

9.1.1 Ethical and legislative advances

The historical context, ethical considerations, and legislative frameworks have been pivotal in shaping the path of xenotransplantation. From early attempts at cross-species transplantation to the contemporary emphasis on animal welfare and informed consent, the field has evolved significantly.

9.1.2 Advancements in genetic engineering

Genetic engineering has ushered in a new era for xenotransplantation, allowing for the creation of genetically modified pigs with organs more compatible with human recipients. These "designer pigs" represent a breakthrough in reducing immunological barriers.

9.1.3 Clinical xenotransplant trials

The initiation of clinical studies involving xeno-hearts, kidneys and islet cells marks a critical step in validating the safety and efficacy of xenotransplantation in humans. These studies can move forward to trials which hold the potential to significantly expand the pool of available organs and improve treatment options for many diseases and conditions.

9.1.4 Patient perspectives

Patients eagerly anticipate the prospects of xenotransplantation, viewing it as a lifeline for lifesaving or life-improving interventions. Understanding and addressing their perspectives and expectations are essential for responsible clinical progress.

9.1.5 Public opinion and media influence

Public opinion and media coverage play a substantial role in shaping the future of xenotransplantation. The media's role in disseminating accurate and balanced information is critical in fostering constructive public dialogue and minimizing misconceptions.

9.2 Continuing ethical and legislative advancements

Ethical and legislative advancements are indispensable as xenotransplantation moves closer to clinical translation. The responsible treatment of animals, transparent informed consent, and comprehensive regulatory frameworks are fundamental to ensuring ethical and safe practices.

9.3 Alleviating the organ shortage crisis

The potential of xenotransplantation to alleviate the organ shortage crisis cannot be overstated. As clinical trials progress and demonstrate the viability of xenotransplantation, it stands as a beacon of hope for those awaiting life-saving organ transplants.

9.4 Promising future for xenotransplantation

The promising future for xenotransplantation lies in its potential to bridge the gap between the demand for organs and their limited supply. With continued collaboration, ethical diligence, and advancements in science, xenotransplantation can move from the realm of theoretical possibility to practical reality.

To bring xenotransplantation to the clinic, the scientific community, regulatory bodies, and the media must work in harmony. International collaboration is essential to continue to establish common standards and guidelines, enabling the field to progress responsibly and ethically in a universal fashion on an international stage.

As we navigate the uncharted frontiers of xenotransplantation and further clinical application, ethical decisions and legislation that accompany it, the future looks promising, provided we remain steadfast in our commitment to science, ethics, and the well-being of both humans and animals. This review underscores the remarkable potential of xenotransplantation while recognizing the importance of treading the path to the clinic with care, diligence, empathy, and informed action including harmonization of guidance's' and legislation internationally.

Author contributions

WH: Conceptualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

I wish to acknowledge the International Xenotransplantation Association (IXA), The IXA Ethics Committee for their ongoing support of the IXA's mission to promote xenotransplantation as a safe, ethical, and effective therapeutic modality by; 1) Fostering the science of xenotransplantation through promotion of ethical clinical and pre-clinical research, productive discourse, and collaboration; 2) Educating health care providers and lay persons through broad, representative participation in interactive public debate; and 3) Guiding the development of scientifically sound, internationally consistent public policy that is responsive to new developments in the field and acknowledges varying social, ethical and legal frameworks.

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.

Publisher's note

References

1. National Academies of Sciences E, Medicine, Health, Medicine D, Board on Health Care S and Board on Health Sciences P, et al. The national academies collection: reports funded by national institutes of health. In: Hackmann M, English RA, Kizer KW, editors. *Realizing the Promise of Equity in the Organ Transplantation System*. Washington (DC: National Academies Press (US (2022).

2. Communiqué TC. First WHO global consultation on regulatory requirements for xenotransplantation clinical trials: Changsha, China, 19-21 November 2008. The Changsha communiqué. *Xenotransplantation* (2009) 16(2):61–3. doi: 10.1111/j.1399-3089.2009.00520.x

3. World Health O. *Second WHO global consultation on regulatory requirements for xenotransplantation clinical trials* (2011). Geneva, Switzerland: World Health Organization. Available at: https://iris.who.int/bitstream/handle/10665/341817/WHO-HTP-EHT-CPR-2011.01-eng.pdf?sequence=1&isAllowed=y (Accessed 20/11/2023).

4. Hawthorne WJ, Cowan PJ, Bühler LH, Yi S, Bottino R, Pierson RN3rd, et al. Third WHO global consultation on regulatory requirements for xenotransplantation clinical trials, Changsha, Hunan, China December 12-14, 2018: "The 2018 Changsha communiqué" The 10-year anniversary of the international consultation on xenotransplantation. *Xenotransplantation* (2019) 26(2):e12513. doi: 10.1111/xen.12513

5. Kwon I, Mo H. Xenotransplantation. In: ten Have H, editor. *Encyclopedia of Global Bioethics*. Cham: Springer International Publishing (2014). p. 1–14.

6. Hawthorne WJ, Thomas A, Pierson RN. Ethics and theoretical issues in kidney xenotransplantation. *Semin Nephrol* (2022) 42(4):151288. doi: 10.1016/j.semnephrol.2022.151288

7. Hawthorne WJ, Cowan PJ, Buhler L, Wolf E. International standards and guidelines for xenotransplantation. *Nat Biotechnol* (2021) 39(12):1501–2. doi: 10.1038/s41587-021-01148-3

8. Schiff T, Parent B, Dittmer I, Hawthorne WJ, Kwon I, Mohiuddin MM. Next steps for clinical xenotransplantation in the United States. *Ann Internal Med* (2023) 176:1538–153. doi: 10.7326/m23-1823%m37903363

9. Salvaris EJ, Fisicaro N, McIlfatrick S, Thomas A, Fuller E, Lew AM, et al. Characterisation of transgenic pigs expressing a human T cell-depleting anti-CD2 monoclonal antibody. *Xenotransplantation* (2023) 13(n/a):e12836. doi: 10.1111/xen.12836

10. Griffith BP, Goerlich CE, Singh AK, Rothblatt M, Lau CL, Shah A, et al. Genetically modified porcine-to-human cardiac xenotransplantation. *New Engl J Med* (2022) 387(1):35–44. doi: 10.1056/NEJMoa2201422

11. Porrett PM, Orandi BJ, Kumar V, Houp J, Anderson D, Cozette Killian A, et al. First clinical-grade porcine kidney xenotransplant using a human decedent model. *Am J Transplant Off J Am Soc Transplant Am Soc Transplant Surgeons* (2022) 22(4):1037–53. doi: 10.1111/ajt.16930

12. Montgomery RA, Stern JM, Lonze BE, Tatapudi VS, Mangiola M, Wu M, et al. Results of two cases of pig-to-human kidney xenotransplantation. *New Engl J Med* (2022) 386(20):1889–98. doi: 10.1056/NEJMoa2120238

13. Hawthorne WJ, Salvaris EJ, Chew YV, Burns H, Hawkes J, Barlow H, et al. Xenotransplantation of genetically modified neonatal pig islets cures diabetes in baboons. *Front Immunol* (2022) 13:898948. doi: 10.3389/fimmu.2022.898948

14. Mohiuddin MM, Singh AK, Scobie L, Goerlich CE, Grazioli A, Saharia K, et al. Graft dysfunction in compassionate use of genetically engineered pig-to-human cardiac xenotransplantation: a case report. *Lancet* (2023) 402(10399):397–410. doi: 10.1016/S0140-6736(23)00775-4

15. dos Santos RMN. Kidney xenotransplantation: are we ready for prime time? *Curr Urol Rep* (2023) 24(6):287–97. doi: 10.1007/s11934-023-01156-7

16. Denys JB. *Lettre escrite à Monsieur de Montmor ... touchant une nouvelle maniere de guerir plusieurs maladies par la transfusion du sang, confirmée par deux experiences faites sur des hommes* (1667). Available at: https://gallica.bnf.fr/ark:/12148/bpt6k326277d (Accessed 20/11/2023).

17. Jaboulay M. Greffe de reins au pli du coude par soudures arterielles et veineuses (1906) (Accessed 23/11/2023).

18. Thomas A, Hawthorne WJ, Burlak C. Xenotransplantation literature update, November/December 2019. *Xenotransplantation* (2020) 27(1):e12582. doi: 10.1111/xen.12582

19. Hu M, Hawthorne WJ, Yi S, O'Connell PJ. Cellular immune responses in islet xenograft rejection. *Front Immunol* (2022) 13:893985. doi: 10.3389/fimmu.2022.893985

20. Carrier AN, Verma A, Mohiuddin M, Pascual M, Muller YD, Longchamp A, et al. Xenotransplantation: A new era. *Front Immunol* (2022) 13:900594. doi: 10.3389/fimmu.2022.900594

21. Cooper DKC, Ekser B, Tector AJ. Immunobiological barriers to xenotransplantation. *Int J Surg (London England)* (2015) 23(Pt B):211–6. doi: 10.1016/j.ijsu.2015.06.068

22. Sykes M. Developing pig-to-human organ transplants. *Sci (New York NY)* (2022) 378(6616):135–6. doi: 10.1126/science.abo7935

23. Cooper DKC, Ekser B, Tector AJ. A brief history of clinical xenotransplantation. *Int J Surg (London England)* (2015) 23(Pt B):205–10. doi: 10.1016/j.ijsu.2015.06.060

24. Denner J. Virus safety of xenotransplantation. *Viruses* (2022) 14(9):1926. doi: 10.3390/v14091926

25. Groenendaal H, Costard S, Ballard R, Bienhoff S, Challen DC, Dominguez BJ, et al. Expert opinion on the identification, risk assessment, and mitigation of microorganisms and parasites relevant to xenotransplantation products from pigs. *Xenotransplantation* (2023) 30(5):e12815. doi: 10.1111/xen.12815

26. Niu D, Wei HJ, Lin L, George H, Wang T, Lee IH, et al. nactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. *Sci (New York NY)* (2017) 357(6357):1303–7. doi: 10.1126/science.aan4187

27. Chen JQ, Zhang MP, Tong XK, Li JQ, Zhang Z, Huang F, et al. Scan of the endogenous retrovirus sequences across the swine genome and survey of their copy number variation and sequence diversity among various Chinese and Western pig breeds. *Zoological Res* (2022) 43(3):423–41. doi: 10.24272/j.issn.2095-8137.2021.379

28. Denner J, Längin M, Reichart B, Krüger L, Fiebig U, Mokelke M, et al. Impact of porcine cytomegalovirus on long-term orthotopic cardiac xenotransplant survival. *Sci Rep* (2020) 10(1):17531. doi: 10.1038/s41598-020-73150-9

29. Morozov VA, Wynyard S, Matsumoto S, Abalovich A, Denner J, Elliott R. No PERV transmission during a clinical trial of pig islet cell transplantation. *Virus Res* (2017) 227:34–40. doi: 10.1016/j.virusres.2016.08.012

30. Saharia KKTeam tUoMX, , Hall VG, Chesdachai S, Porrett P, Fishman JA, et al. Heart of the matter—infection and xenotransplantation. *Transpl Infect Dis* (2023) 2023(n/a):e14206. doi: 10.1111/tid.14206

31. Hawthorne WJ. Partnership of the International Xenotransplantation Association, The Transplantation Society, and World Health Organization in the development of guidance documents and policies for xenotransplantation including the Changsha communiqué. *Xenotransplantation* (2020) 27(3):e12604. doi: 10.1111/xen.12604

32. Europe Co. *Explanatory Report to recommendation Rec (2003) 10 of the Committee of Ministers to Member States on xenotransplantation, Article 21* (2003). Available at: https://www.coe.int/t/dg3/healthbioethic/Activities/06_Xenotransplantation_en/INF_2003_12exenoER.pdf (Accessed 17/01/2024).

33. Code US. PART 70 - INTERSTATE QUARANTINE. In: *Title 42 - public health* (2012). Available at: https://www.govinfo.gov/content/pkg/CFR-2013-title42-vol1/xml/CFR-2013-title42-vol1-part70.xml (Accessed 17/01/2024).

34. Denner J. Can antiretroviral drugs be used to treat porcine endogenous retrovirus (PERV) infection after xenotransplantation? *Viruses* (2017) 9(8):213. doi: 10.3390/v9080213

35. Gyngell C, Munsie M, Fujita M, Thiessen C, Savulescu J, Konstantinov IE. Ethical analysis of the first porcine cardiac xenotransplantation. *J Med Ethics* (2023) jme-2022:108685. doi: 10.1136/jme-2022-108685

36. Hurst DJ, Padilla L, Paris WD. *Xenotransplantation ethical, regulatory, and social aspects*. New York, USA: Springer Cham (2023). doi: 10.1007/978-3-031-29071-8

37. (IXA) TTS. Regulatory documents and guidelines, in: *The Transplantation Society* . Available at: https://tts.org/79-ixa/ixa-resources/123-ixa-reg-guidelines (Accessed 20/11/2023).

38. Goralnik L, Nelson MP. Anthropocentrism. In: *Encyclopedia of Applied Ethics, 2nd ed*. Chadwick R, editor. (2012). Academic Press. p. 145–55. doi: 10.1016/B978-0-12-373932-2.00349-5

39. Shaw D, Dondorp W, De Wert G. Ethical issues surrounding the transplantation of organs from animals into humans. *Rev scientifique technique (International Office Epizootics)* (2018) 37(1):123–9. doi: 10.20506/rst.37.1.2745

40. Hawthorne WJ. World first pig-to-human cardiac xenotransplantation. *Xenotransplantation* (2022) 29(1):e12733. doi: 10.1111/xen.12733

41. Rollin BE. Ethical and societal issues occasioned by xenotransplantation. *Anim (Basel)* (2020) 10(9):1695. doi: 10.3390/ani10091695

42. FDA. *Source Animal, Product, Preclinical, and Clinical Issues Concerning the Use of Xenotransplantation Products in Humans* (2016). Available at: https://www.fda.gov/files/vaccines,%20blood%20&%20biologics/published/Source-Animal–Product–Preclinical–and-Clinical-Issues-Concerning-the-Use-of-Xenotransplantation-Products-in-Humans–Guidance-for-Industry.pdf (Accessed 20/11/2023).

43. (EMA) EMA. *GUIDELINE ON XENOGENEIC CELL-BASED MEDICINAL PRODUCTS* (2009) (Accessed 20/11/2023).

44. TTS-ISN. The declaration of Istanbul on organ trafficking and transplant tourism (2018 Edition). *Transplantation* (2019) 103(2):218-9. doi: 10.1097/TP.0000000000002540

45. Fishman JA, Scobie L, Takeuchi Y. Xenotransplantation-associated infectious risk: a WHO consultation. *Xenotransplantation* (2012) 19(2):72–81. doi: 10.1111/j.1399-3089.2012.00693.x

46. Hawthorne WJ, Cowan PJ. Xenotransplantation in Australia: Development of the regulatory process. *Xenotransplantation* (2020) 27(3):e12603. doi: 10.1111/xen.12603

47. Code US. *Sec. 262 - Regulation of biological products. (2010);Title 42 - THE PUBLIC HEALTH AND WELFARE. CHAPTER 6A - PUBLIC HEALTH SERVICE. SUBCHAPTER II - GENERAL POWERS AND DUTIES. Part F - Licensing of Biological Products and Clinical Laboratories. subpart 1 - biological products* . Available at: https://www.govinfo.gov/content/pkg/USCODE-2010-title42/html/USCODE-2010-title42-chap46.htm (Accessed 17/01/2024).

48. Denner J. Recent progress in xenotransplantation, with emphasis on virological safety. *Ann Transplant* (2016) 21:717–27. doi: 10.12659/aot.900531

49. Buhler L, Hawthorne WJ. International xenotransplantation association (IXA) 25th anniversary. *Xenotransplantation* (2023) 30(4):e12821. doi: 10.1111/xen.12821

50. Mohiuddin MM, Singh AK, Goerlich CE. Preclinical rationale and current pathways to support the first human clinical trials in cardiac xenotransplantation. *Hum Immunol* (2023) 84(1):34–42. doi: 10.1016/j.humimm.2022.07.001

51. Locke JE, Kumar V, Anderson D, Porrett PM. Normal graft function after pig-to-human kidney xenotransplant. *JAMA Surg* (2023) 158(10):1106–8. doi: 10.1001/jamasurg.2023.2774

52. Moazami N, Stern JM, Khalil K, Kim JI, Narula N, Mangiola M, et al. Pig-to-human heart xenotransplantation in two recently deceased human recipients. *Nat Med* (2023) 29(8):1989–97. doi: 10.1038/s41591-023-02471-9

53. Loupy A, Goutaudier V, Giarraputo A, Mezine F, Morgand E, Robin B, et al. Immune response after pig-to-human kidney xenotransplantation: a multimodal phenotyping study. *Lancet* (2023) 402(10408):1158–69. doi: 10.1016/S0140-6736(23)01349-1

54. Tector AJ, Adams AB, Tector M. Current status of renal xenotransplantation and next steps. *Kidney360* (2023) 4(2):278–84. doi: 10.34067/kid.0007152021

55. Heneine W, Tibell A, Switzer WM, Sandstrom P, Rosales GV, Mathews A, et al. No evidence of infection with porcine endogenous retrovirus in recipients of porcine islet-cell xenografts. *Lancet (London England)* (1998) 352(9129):695–9. doi: 10.1016/s0140-6736(98)07145-1

56. Wang W, Mo Z, Ye B, Hu P, Liu S, Yi S. A clinical trial of xenotransplantation of neonatal pig islets for diabetic patients. *Zhong nan da xue xue bao Yi xue ban = J Cent South Univ Med Sci* (2011) 36(12):1134–40. doi: 10.3969/j.issn.1672-7347.2011.12.002

57. Ekser B, Cooper DK. Overcoming the barriers to xenotransplantation: prospects for the future. *Expert Rev Clin Immunol* (2010) 6(2):219–30. doi: 10.1586/eci.09.81

58. Rayat GR, Gazda LS, Hawthorne WJ, Hering BJ, Hosking P, Matsumoto S, et al. First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes–Chapter 3: Porcine islet product manufacturing and release testing criteria. *Xenotransplantation* (2016) 23(1):38–45. doi: 10.1111/xen.12225

59. Arefanian H, Ramji Q, Gupta N, Spigelman AF, Grynoch D, MacDonald PE, et al. Yield, cell composition, and function of islets isolated from different ages of neonatal pigs. *Front Endocrinol (Lausanne)* (2022) 13:1032906. doi: 10.3389/fendo.2022.1032906

60. Baliker M, VR G. Patient perspective on xenotransplantation. *Kidney360* (2022) 3(11):1953–4. doi: 10.34067/kid.0003542022

61. Paris W, Seidler RJH, FitzGerald K, Padela AI, Cozzi E, Cooper DKC. Jewish, Christian and Muslim theological perspectives about xenotransplantation. *Xenotransplantation* (2018) 25(3):e12400. doi: 10.1111/xen.12400

62. Kreis J, Schmidt H. Public engagement in health technology assessment and coverage decisions: a study of experiences in France, Germany, and the United Kingdom. *J Health politics Policy Law* (2013) 38(1):89–122. doi: 10.1215/03616878-1898812

63. Henderson ML, Adler JT, Van Pilsum Rasmussen SE, Thomas AG, Herron PD, Waldram MM, et al. How should social media be used in transplantation? A survey of the american society of transplant surgeons. *Transplantation* (2019) 103(3):573–80. doi: 10.1097/tp.0000000000002243

64. Cooper DK, Ekser B, Ramsoondar J, Phelps C, Ayares D. The role of genetically engineered pigs in xenotransplantation research. *J Pathol* (2016) 238(2):288–99. doi: 10.1002/path.4635

65. Xi J, Zheng W, Chen M, Zou Q, Tang C, Zhou X. Genetically engineered pigs for xenotransplantation: Hopes and challenges. *Front Cell Dev Biol* (2023) 10:1093534. doi: 10.3389/fcell.2022.1093534

66. Cozzi E, Bosio E, Seveso M, Rubello D, Ancona E. Xenotransplantation as a model of integrated, multidisciplinary research. *Organogenesis* (2009) 5(1):14–22. doi: 10.4161/org.7578

67. WHO. *Strengthening global health security at the human-animal interface* . Available at: https://www.who.int/activities/strengthening-global-health-security-at-the-human-animal-interface (Accessed 20/11/2023).

68. Cooper DKC, Pierson RN 3rd, Hering BJ, Mohiuddin MM, Fishman JA, Denner J, et al. Regulation of clinical xenotransplantation-time for a reappraisal. *Transplantation* (2017) 101(8):1766–9. doi: 10.1097/tp.0000000000001683

69. Hu X, Geng Z, Gonelle-Gispert C, Hawthrone WJ, Deng S, Buhler L. International human xenotransplantation inventory: A 10-year follow-up. *Transplantation* (2022) 106(9):1713–6. doi: 10.1097/tp.0000000000004016

frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 27 February 2024
DOI 10.3389/fimmu.2024.1351717

OPEN ACCESS

EDITED BY
Burcin Ekser,
Indiana University Bloomington, United States

REVIEWED BY
Yi Wang,
Second Affiliated Hospital of Hainan Medical University, China
Suzanne Bertera,
Allegheny General Hospital, United States
Melanie L. Graham,
University of Minnesota Twin Cities, United States

*CORRESPONDENCE
Kazuhiko Yamada
kyamada6@jhmi.edu

RECEIVED 06 December 2023
ACCEPTED 08 February 2024
PUBLISHED 27 February 2024

CITATION
Eisenson DL, Iwase H, Chen W, Hisadome Y, Cui W, Santillan MR, Schulick AC, Gu D, Maxwell A, Koenig K, Sun Z, Warren D and Yamada K (2024) Combined islet and kidney xenotransplantation for diabetic nephropathy: an update in ongoing research for a clinically relevant application of porcine islet transplantation. *Front. Immunol.* 15:1351717. doi: 10.3389/fimmu.2024.1351717

Combined islet and kidney xenotransplantation for diabetic nephropathy: an update in ongoing research for a clinically relevant application of porcine islet transplantation

Daniel L. Eisenson[1], Hayato Iwase[1], Weili Chen[1], Yu Hisadome[1], Wanxing Cui[2], Michelle R. Santillan[1], Alexander C. Schulick[1], Du Gu[1], Amanda Maxwell[3], Kristy Koenig[1], Zhaoli Sun[1], Daniel Warren[1] and Kazuhiko Yamada[1]*

[1]Department of Surgery, The Johns Hopkins University School of Medicine, Baltimore, MD, United States, [2]Cell Therapy and Manufacturing, Medstar Georgetown University Hospital, Washington DC, United States, [3]Research Animal Resources, The Johns Hopkins University School of Medicine, Baltimore, MD, United States

Combined islet and kidney xenotransplantation for the treatment of diabetic nephropathy represents a compelling and increasingly relevant therapeutic possibility for an ever-growing number of patients who would benefit from both durable renal replacement and cure of the underlying cause of their renal insufficiency: diabetes. Here we briefly review immune barriers to islet transplantation, highlight preclinical progress in the field, and summarize our experience with combined islet and kidney xenotransplantation, including both challenges with islet-kidney composite grafts as well as our recent success with sequential kidney followed by islet xenotransplantation in a pig-to-baboon model.

KEYWORDS

islet xenotransplantation, islet-kidney, xenogeneic immune response, tolerance, xenotransplantation

Abbreviations: ELISpot, enzyme-linked immunosorbent spot assay; GalTKO, α-1,3 Galactosyl transferase gene knockout; hCD55, human CD55; hCD59, human CD59; hCD47, human CD47; IBMIR, instant blood-mediated inflammatory reaction; I-K, islet-kidney; IEQ, islet equivalents; IFN-γ, interferon gamma; IPN, islet particle number; mAb, monoclonal antibody; Nab, natural antibody; NHP, non-human primate; NK, natural killer; PTFE, PolyTetraFluoroEthylene; VTL, vascularized thymic lobe.

Introduction

Diabetes is a leading cause of both cardiovascular disease and end stage renal disease (ESRD), and incidence is increasing across the country and across the globe (1). Human islet transplantation is an effective treatment for diabetic patients but requires lifelong immunosuppression: prospective islet transplant recipients must weigh the risks of immunosuppression against the short- and long-term complications of diabetes. Patients with diabetic nephropathy represent a unique – and growing – population that would benefit from both islet and kidney transplantation. Indeed, the favorable risk-to-benefit considerations of combined islet and kidney transplantation in this population inspired recent promising clinical studies in islet after kidney transplantation led by the Clinical Islet Transplantation (CIT) Consortium (2). However, at present these procedures are rare, due, in part, to a shortage of deceased donor organs (3). Xenotransplantation using organs derived from pigs may overcome this organ shortage and allow for broader application of combined islet and kidney transplantation.

The past several years have seen enormous progress in the field of xenotransplantation, with advances in gene-editing and immunosuppression leading to long-term survivals of both kidney and heart xenografts in pig-to-nonhuman primate (NHP) studies (4–7), as well as early studies (preclinical and clinical) in humans (8–10). Clinical translation of porcine islet transplantation predated these recent successes in solid organ xenotransplantation, with encouraging pig-to-NHP studies leading to several small clinical studies using porcine islets in humans (11–21). However, results of these early studies in clinical islet xenotransplantation have been mixed. While these differences between outcomes of preclinical and clinical xenogeneic islet transplantation may be partly explained by differences in the immunosuppression regimens used in the clinical trials – notably, CD40/CD40L costimulatory blockade, which has been critical to success in most preclinical studies, was not utilized – further trials have been limited by more recent consensus guidelines outlining an international framework to promote standardized clinical translation of pig-to-human islet transplantation from source pig development and manufacturing to patient monitoring (22).

Moreover, in the many years since these clinical studies in islet xenotransplantation were conducted, the landscape of diabetes management has changed. Patients with diabetes have other options for durable disease management. Innovations in glucose monitoring and the rapid development of hybrid closed-loop insulin delivery systems have improved quality of life for patients living with diabetes (23), and ongoing clinical trials of novel stem-cell derived islet cell therapy have published early and highly promising results (24). However, porcine islet xenotransplantation remains a compelling therapeutic possibility for patients with diabetic nephropathy who need both kidney and islet replacement. In these patients, there are minimal added risks associated with islet transplantation, as these patients are already on immunosuppression for their kidney grafts; in fact, islets may help protect against premature kidney graft loss associated with diabetes (25) as well as improve long-term vascular diabetic outcomes (26). Here, we will briefly highlight immunologic barriers in porcine islet transplantation, chronicle preclinical progress in the field, and summarize our own experience in combined islet and kidney transplantation, using both 1) vascularized islets in an islet-kidney composite graft, and 2) our more recent strategy of sequential kidney followed by islet xenotransplantation.

Immunologic barriers in pig-to-primate islet xenotransplantation

Porcine xenografts, including pig islets, elicit robust immune responses in humans. These responses involve both innate barriers (27) – including preformed natural antibodies (Nabs) and species incompatibilities in complement and coagulation systems leading to dysregulation – and adaptive immune components (reviewed in (28)). As with transplantation of solid organs, humoral immunity remains a key obstacle to long-term xenograft survival, and T cell-targeted immunosuppression strategies have been critical for prolonging islet survival (20, 29),. Unlike transplantation of other organs, however, transplanted islets also trigger an immediate inflammatory response, known as instant blood-mediated inflammatory reaction (IBMIR) (30, 31), related to expression of tissue factor on islets and leading to activation of innate responses that subsequently consume islets (32, 33). Although IBMIR is seen in auto- and allogeneic islet transplantation, greater immune barriers in xenotransplantation may lead to more pronounced islet losses (34, 35) as high as 70% in some studies (36).

Preclinical progress in porcine islet xenotransplantation: encapsulation and source pig genetic modifications

Various strategies have been developed to overcome these short- and long-term immunologic hurdles, including islet encapsulation and source pig genetic modifications – both of which are intended to reduce the immunogenicity of the porcine islets.

Broadly, islet encapsulation technologies include microencapsulation of islets in alginate matrix, and macro-encapsulation of immobilized islets in bi-layered PTFE with a common oxygenation chamber (37). This microencapsulation technique successfully reversed diabetes for up to six months in preclinical studies of rhesus macaques (38), and was subsequently used in two nationally regulated clinical studies of porcine islet xenotransplantation in New Zealand and Argentina. Follow-up studies confirmed modest clinical benefit including reduction in HbA1c, hospitalization, and severe hypoglycemic and/or hyperglycemic events (21, 39) The key advantage of these technologies is that encapsulation may protect islets from the recipient immune system and obviate the need for immunosuppression; whereas islet transplantation alone is currently reserved for patients with hypoglycemic unawareness due to the morbidity of immunosuppression, transplantation of encapsulated islets without immunosuppression may tilt the risk-

benefit ratio in favor of islet transplantation for cure of diabetes. Still, important hurdles remain in broader clinical application of this technology including variable recipient immune responses to the encapsulation material, which may lead to fibrosis of encapsulated grafts.

Source pig genetic modification is another strategy to overcome innate immune barriers and can be divided into two major categories: elimination of carbohydrate antigens that are targets of preformed antibodies, and correction of species incompatibilities. In solid organ xenotransplantation, preformed antibody binding leads to hyperacute rejection of graft; in free islet xenotransplantation, preformed antibody binding leads to an amplified IBMIR with islet loss (40). While elimination of targets of preformed Nabs (particularly elimination of *α-gal* with creation of α-1,3 Galactosyl transferase gene knockout or GalTKO source pigs) has been essential for successful pig-to-NHP heart and kidney xenotransplantation (4, 41, 42), the impact of using GalTKO source pigs on xenograft survival in islet transplantation is less conclusive, which may be a function of changes in α-gal expression with islet maturation (43–45). Similarly, correcting for species incompatibilities between porcine and primate complement regulatory systems through individual insertion of human complement regulatory proteins may not significantly reduce the incidence of IBMIR (44). However, combining carbohydrate antigen gene knockouts with complement regulatory transgenes proves additive: xenogeneic islets from GalTKO.hCD55.hCD59 and GalTKO.hCD39.hCD46 source pigs demonstrated reduced islet loss and attenuated IBMIR (15, 16). More recently, islets derived from neonatal GalTKO.hCD55.hCD59 source pigs demonstrated cure of diabetes with >1 year of insulin independence in the stringent pig-to-baboon model (46).

Combined kidney and islet xenotransplantation to broaden clinical applicability of porcine islet xenotransplantation

Marked improvements in diabetes management and emerging therapies have changed the risk-benefit calculus associated with islet transplantation more broadly, and porcine islet xenotransplantation in particular. As described in the preceding section, encapsulation technologies – which may allow for durable glucose control without immunosuppression – remain one relevant application for porcine islet xenotransplantation. Another relevant strategy is combining porcine islet xenotransplantation with solid organ xenotransplantation. This strategy has already been employed with success by the CIT consortium treating diabetic nephropathy with islet transplantation after kidney transplantation, but broader application is limited by the shortage of deceased donor organs. The following sections detail our preclinical experience with combined islet and kidney transplantation, including both composite islet-kidney transplantation as well as kidney-first sequential islet and kidney xenotransplantation.

Combined islet and kidney xenotransplantation: our experience with composite islet-kidney xenotransplantation

Composite islet-kidney transplantation for cure of diabetic nephropathy: concept and supporting allogeneic data

As detailed above, xenogeneic islets are susceptible to destruction by both innate and adaptive mechanisms. The senior author of this review has demonstrated that transplanting pre-vascularized islets as part of a composite organ protects islets from innate immune destruction by circumventing the typical pathway that triggers IBMIR (47–49). We have previously reported successful preparation of composite islet-kidney (I-K) grafts which maintained normoglycemia and normal renal function after transplantation in pig-to-pig and nonhuman primate allogeneic transplantation models (Figure 1). Islets are isolated and pre-vascularized under autologous renal capsule, with subsequent transplantation of composite I-K graft (50). Preclinical allotransplantation studies in both pigs and in NHPs have demonstrated that this procedure preserves islets, likely by limiting innate immune destruction: diabetes is cured in animals who undergo composite I-K transplantation, while animals who undergo conventional free islet injection with the same islet equivalents (IEQs) remain insulin dependent (51, 52). Additional preclinical studies have demonstrated further improvements in islet yield and function with islet protective strategies including siRNA silencing of apoptotic genes (53). However, practical challenges have limited successful translation of this composite organ strategy for cure of diabetes and kidney failure in pig-to-NHP transplantation.

Challenges with translation of allogeneic results to xenogeneic pig-to-non-human primate model

While I-K composite organs are ideally created in the same animal with autologous pig islets transplanted under autologous renal capsule, size constraints in our NHP recipient prevent successful I-K transplantation using a single source pig: a small (<30kg) source pig is needed for successful pig-to-NHP kidney transplantation, while a large (>60kg) source pig is needed in order to obtain sufficient islets for reversal of diabetes. This is a limitation primarily in our preclinical pig-to-NHP model, as larger kidneys from size matched >60kg pigs will likely be appropriate for human adult recipients. Nevertheless, overcoming this experimental constraint is critical to demonstration of composite I-K success. Our own attempts to isolate islets from juvenile source pig pancreases recapitulated the work of other investigators, confirming low islet-equivalent yield from juvenile pigs (54, 55). Accordingly, we elected to use two different source pigs for composite organ creation: large pigs would be used for islets, pre-vascularized prior to transplantation under the renal capsule of a smaller pig.

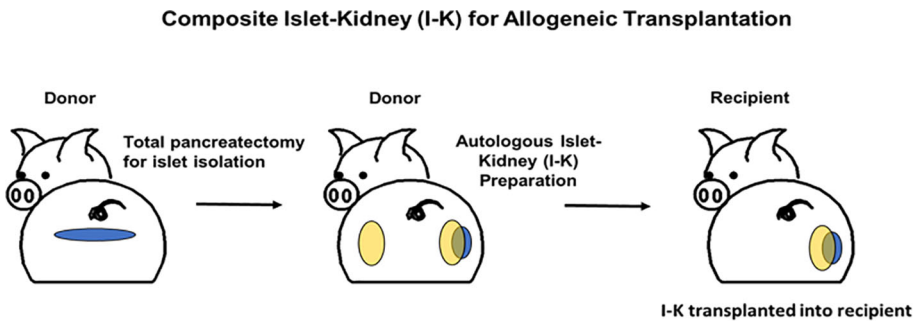


FIGURE 1
Schematic diagrams of preparation of a composite islet-kidney graft in donor and allogeneic composite islet-kidney transplantation in a recipient.

Using different source pigs for I-K composite organ creation introduced other challenges. Allogeneic islets from one source pig transplanted under the renal capsule of another source pig are vulnerable to recipient source pig immune responses, as with any allogeneic transplant. Strategies to mitigate these responses include 1) use of related pairs (cloned, inbred, or MHC-matched), and 2) minimization of the islet pre-vascularization period under allogeneic renal capsule.

Using related pairs for composite islet-kidney creation

Although the use of genetically identical (cloned) pairs for islet-kidney creation would be ideal, previous experiments have demonstrated long-term survival of skin and heart grafts without immunosuppression in highly inbred swine (56). Indeed, inbred animals, defined by co-ancestry >0.9, accepted allogeneic skin grafts for >340 days and accepted allogeneic heart grafts for >265 days without immunosuppression. MHC-matched pairs (co-ancestry >0.75) also allowed for acceptance of kidney grafts without immunosuppression, although hearts and islets were not accepted (50, 56, 57). Over the last three years, we have optimized composite I-K preparation using MHC-matched source pigs. As opposed to autologous I-K preparation, successful allogeneic preparation requires immunosuppression with both high dose tacrolimus and MMF. I-K preparation may be further optimized with reduction in pre-vascularization period from 6 weeks to 2 weeks. Still, it remains unclear whether I-K preparation in MHC-matched pairs preserves sufficient islets for reversal of diabetes in a xenogeneic recipient. We plan to revisit the composite I-K strategy in xenotransplantation when cloned pigs or highly inbred GalTKO pigs are available for these experiments.

Combined islet and kidney xenotransplantation: recent success with sequential kidney followed by islet xenotransplantation

Definitive evaluation of composite I-K transplantation in a pig-to-NHP model also requires a control: independent kidney and free islet transplantation. Negative controls were present in previous studies of composite IK technologies – in pig-to-pig, baboon-to-baboon, and macaque-to-macaque models – and demonstrated preservation of islets with composite I-K transplantation across allogeneic barriers. In the past year, due to lack of inbred or cloned source pigs, we tested an alternative strategy for combined islet and kidney transplantation across a xenogeneic barrier that would also serve as a control of the composite I-K strategy. This alternative approach involves delayed islet transplantation after kidney and vascularized thymus transplantation (role of vascularized thymus transplantation in the induction of tolerance across xenogeneic barriers reviewed in (28)), using a recipient size-matched kidney and thymus source pig, as well as a large source pig for islets. Notably this approach (without thymus co-transplantation) is also similar to recent work in human islet-after-kidney transplantation conducted by the CIT consortium. Although additional cases are required, we have achieved reversal of diabetes and life-supporting renal function for 180 days with this kidney-first sequential islet and kidney xenotransplantation (52). To our knowledge, this is the first demonstration of maintenance of durable normoglycemia and stable creatinine with porcine kidney and islets in a diabetic and life-supporting pig-to-baboon combined kidney, vascularized thymus and islet xenotransplantation model. These preliminary results were recently presented at the International Xenotransplantation Association Congress (San Diego, 2023), and are described in detail in the following sections:

Methods: source pigs, recipient baboons, immunosuppression regimen, and transplantation procedures

In this experiment, we used two GalTKO.hCD55 source pigs from the National Swine Resource and Research Center (*Sus scrofa domesticus*, source: University of Missouri-Columbia, Columbia, MO) and one baboon recipient from the National Research and Resources Program (MD Anderson, Houston TX). Baboon recipient underwent B and T cell depletion with rituximab and rabbit anti-thymocyte globulin, followed by maintenance immunosuppression with anti-CD40 mAb (Nonhuman Primate Reagent Resource, University of Massachusetts Medical School,

Worcester, MA). The baboon received kidney and vascularized thymic lobe grafts from a GalTKO.hCD55 (11.7kg) source pig on POD 0 with bilateral native nephrectomy. Diabetes was subsequently induced with streptozosin (STZ. 100mg/kg on POD 5, 50mg/kg on POD 9). After confirmation of diabetes, baboon underwent free islet transplantation into the portal vein, with islets isolated from unrelated GalTKO.hCD55 (95kg) source pig. Islet isolation was performed as previously described (58) and yielded 101K islet equivalents (IEQs) and 194K islet particle number (IPN). All animals were used in compliance with guidelines provided by the Animal Care and Use Committee at The Johns Hopkins University School of Medicine.

Results: islet-after-kidney pig-to-baboon xenotransplantation cures diabetes and renal insufficiency

Both renal insufficiency and diabetes were cured with kidney-first sequential islet and kidney xenotransplantation. The baboon recipient maintained normal serum creatinine with no evidence of rejection for six months following kidney and islet transplant but was euthanized due to sepsis related to pyelonephritis in setting of stent occlusion on POD180. Immediately after islet transplantation, hyperglycemia was reversed with normalization of blood sugars from >250mg/dL (pre-transplant) to 80-110 mg/dL. Porcine islets functioned and maintained normal BG levels without any exogenous insulin treatment throughout the recipient's postoperative course. Post-mortem evaluation of liver confirmed presence of insulin-staining islets.

Discussion: timing of sequential transplants and immunomodulatory strategies may be important for success of islet-after-kidney xenotransplantation

As referenced above (see *Preclinical progress in porcine islet xenotransplantation*), investigators have recently achieved cure of diabetes in baboons using pooled islets from neonatal genetically modified pig donors (46). However, this required an average of 14 neonatal pancreases (70 piglets for 5 baboon recipients). In our model, we have achieved normoglycemia using islets derived from a single source adult pig with an administered islet mass of 12,500 IEQ/kg. Of note, this is within range though slightly less than was required in the recent clinical islet-after-kidney transplantation studies where successful islet transplants averaged >16,000 IEQ/kg (2).

One reason for the success of free islet transplantation in this model may be timing of sequential transplants: kidney-first transplantation promotes absorption of anti-pig antibodies, likely reducing IBMIR following islet transplantation, corresponding to reduced loss of islets. This may have enabled durable reversal of diabetes with fewer islet equivalents as compared with clinical islet-after-kidney transplantation. Indeed, the possible antibody absorption benefits of sequential transplant timing is less clear in the clinical islet-after-kidney studies, where islet transplantation occurred well after index kidney transplantation.

Lastly, adjunctive immunomodulatory strategies may also have played a role in the durable xenograft survival in this case. This animal received vascularized thymic lobe (VTL) graft co-transplantation from the kidney source pig, which has been shown to prolong xenograft survival in pig-to-baboon renal xenotransplantation (reviewed in (28)). Interferon gamma (IFN-γ) enzyme-linked immunosorbent spot (ELISpot) assay was performed to assess the potential immunomodulatory effect of VTL co-transplantation in this case. ELISpot assay at POD 180 demonstrated pig-specific unresponsiveness, suggesting that co-transplanted VTL graft may promote immunomodulatory effects. Further studies will clarify the mechanisms of *in vitro* unresponsiveness (59).

Additional cases are needed to replicate this work, but these encouraging results indicate that our negative control strategy, sequential kidney followed by islet xenotransplantation may reverse diabetes and renal insufficiency.

Porcine islet xenotransplantation: the best path forward may be dual indication transplantation

Porcine islet xenotransplantation is one promising strategy for cure of diabetes among an evolving landscape of emerging therapies in diabetes management. While islet-alone xenotransplantation strategies continue to show improvement with source pig genetic modifications and refinements to immunosuppression regimens, approaches like encapsulation which allow for reversal of diabetes without immunosuppression may be more clinically relevant. Porcine islet xenotransplantation, in conjunction with kidney xenotransplantation, remains a particularly compelling therapeutic possibility for patients with diabetic nephropathy who require both kidney and islet replacement, and who have already committed to immunosuppression for their kidney grafts. Composite islet-kidney transplantation has proven challenging in xenogeneic preclinical models; however, preliminary studies in islet-after-kidney xenotransplantation are promising and may point to a path forward with combined islet and kidney transplantation for diabetic nephropathy.

Author contributions

DE: Conceptualization, Formal Analysis, Investigation, Methodology, Writing – original draft. HI: Data curation, Formal Analysis, Investigation, Writing – review & editing. WLC: Formal Analysis, Methodology, Writing – review & editing. YH: Formal Analysis, Investigation, Writing – review & editing. WXC: Methodology, Writing – review & editing. MS: Formal

Analysis, Investigation, Writing – review & editing. AS: Investigation, Writing – review & editing. DG: Methodology, Writing – review & editing. AM: Investigation, Writing – review & editing. KK: Methodology, Project administration, Writing – review & editing. ZS: Methodology, Supervision, Writing – review & editing. DW: Investigation, Methodology, Project administration, Writing – review & editing. KY: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

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.

Publisher's note

References

1. Divers J, Mayer-Davis EJ, Lawrence JM, Isom S, Dabelea D, Dolan L, et al. Trends in incidence of type 1 and type 2 diabetes among youths - selected counties and Indian reservations, United States, 2002-2015. *MMWR Morb Mortal Wkly Rep*. (2020) 69:161–5. doi: 10.15585/mmwr.mm6906a3

2. Markmann JF, Rickels MR, Eggerman TL, Bridges ND, Lafontant DE, Qidwai J, et al. Phase 3 trial of human islet-after-kidney transplantation in type 1 diabetes. *Am J Transplant*. (2021) 21:1477–92. doi: 10.1111/ajt.16174

3. Gamble A, Pepper AR, Bruni A, Shapiro AMJ. The journey of islet cell transplantation and future development. *Islets*. (2018) 10:80–94. doi: 10.1080/19382014.2018.1428511

4. Rivard CJ, Tanabe T, Lanaspa MA, Watanabe H, Nomura S, Andres-Hernando A, et al. Upregulation of CD80 on glomerular podocytes plays an important role in development of proteinuria following pig-to-baboon xeno-renal transplantation - an experimental study. *Transpl Int*. (2018) 31:1164–77. doi: 10.1111/tri.13273

5. Adams AB, Lovasik BP, Faber DA, Burlak C, Breeden C, Estrada JL, et al. Anti-C5 antibody tesidolumab reduces early antibody-mediated rejection and prolongs survival in renal xenotransplantation. *Ann Surg*. (2021) 274:473–80. doi: 10.1097/SLA.0000000000004996

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

7. Mohiuddin MM, Goerlich CE, Singh AK, Zhang T, Tatarov I, Lewis B, et al. Progressive genetic modifications of porcine cardiac xenografts extend survival to 9 months. *Xenotransplantation*. (2022) 29:e12744. doi: 10.1111/xen.12744

8. Griffith BP, Goerlich CE, Singh AK, Rothblatt M, Lau CL, Shah A, et al. Genetically modified porcine-to-human cardiac xenotransplantation. *N Engl J Med*. (2022) 387:35–44. doi: 10.1056/NEJMoa2201422

9. Montgomery RA, Stern JM, Lonze BE, Tatapudi VS, Mangiola M, Wu M, et al. Results of two cases of pig-to-human kidney xenotransplantation. *N Engl J Med*. (2022) 386:1889–98. doi: 10.1056/NEJMoa2120238

10. Locke JE, Kumar V, Anderson D, Porrett PM. Normal graft function after pig-to-human kidney xenotransplant. *JAMA Surg*. (2023). doi: 10.1001/jamasurg.2023.2774

11. Groth CG, Korsgren O, Tibell A, Tollemar J, Moller E, Bolinder J, et al. Transplantation of porcine fetal pancreas to diabetic patients. *Lancet*. (1994) 344:1402–4. doi: 10.1016/s0140-6736(94)90570-3

12. Valdes-Gonzalez R, Rodriguez-Ventura AL, White DJ, Bracho-Blanchet E, Castillo A, Ramirez-Gonzalez B, et al. Long-term follow-up of patients with type 1 diabetes transplanted with neonatal pig islets. *Clin Exp Immunol*. (2010) 162:537–42. doi: 10.1111/j.1365-2249.2010.04273.x

13. Valdes-Gonzalez RA, Dorantes LM, Garibay GN, Bracho-Blanchet E, Mendez AJ, Davila-Perez R, et al. Xenotransplantation of porcine neonatal islets of Langerhans and Sertoli cells: a 4-year study. *Eur J Endocrinol*. (2005) 153:419–27. doi: 10.1530/eje.1.01982

14. Rood PP, Bottino R, Balamurugan AN, Smetanka C, Ayares D, Groth CG, et al. Reduction of early graft loss after intraportal porcine islet transplantation in monkeys. *Transplantation*. (2007) 83:202–10. doi: 10.1097/01.tp.0000250680.36942.c6

15. Bottino R, Wijkstrom M, Windt der van DJ, Hara H, Ezzelarab M, Murase N, et al. Pig-to-monkey islet xenotransplantation using multi-transgenic pigs. *Am J Transplant*. (2014) 14:2275–87. doi: 10.1111/ajt.12868

16. Hawthorne WJ, Salvaris EJ, Phillips P, Hawkes J, Liuwantara D, Burns H, et al. Control of IBMIR in neonatal porcine islet xenotransplantation in baboons. *Am J Transplant*. (2014) 14:1300–9. doi: 10.1111/ajt.12722

17. Shin JS, Kim JM, Min BH, Yoon IH, Kim HJ, Kim JS, et al. Pre-clinical results in pig-to-non-human primate islet xenotransplantation using anti-CD40 antibody (2C10R4)-based immunosuppression. *Xenotransplantation*. (2018) 25. doi: 10.1111/xen.12356

18. Min BH, Shin JS, Kim JM, Kang SJ, Kim HJ, Yoon IH, et al. Delayed revascularization of islets after transplantation by IL-6 blockade in pig to non-human primate islet xenotransplantation model. *Xenotransplantation*. (2018) 25. doi: 10.1111/xen.12374

19. Cardona K, Korbutt GS, Milas Z, Lyon J, Cano J, Jiang W, et al. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat Med*. (2006) 12:304–6. doi: 10.1038/nm1375

20. Hering BJ, Wijkstrom M, Graham ML, Hardstedt M, Aasheim TC, Jie T, et al. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. *Nat Med*. (2006) 12:301–3. doi: 10.1038/nm1369

21. Matsumoto S, Abalovich A, Wechsler C, Wynyard S, Elliott RB. Clinical benefit of islet xenotransplantation for the treatment of type 1 diabetes. *EBioMedicine*. (2016) 12:255–62. doi: 10.1016/j.ebiom.2016.08.034

22. Hering BJ, Wijkstrom M, Graham ML, Hardstedt M, Aasheim TC, Jie T, et al. First update of the International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes–Executive summary. *Xenotransplantation*. (2016) 23:3–13. doi: 10.1111/xen.12231

23. Templer S. Closed-loop insulin delivery systems: past, present, and future directions. *Front Endocrinol (Lausanne)*. (2022) 13:919942. doi: 10.3389/fendo.2022.919942

24. Reichman TW, Ricordi C, Naji A, Markmann JF, Perkins BA, Wijkstrom M, et al. 836-P: glucose-dependent insulin production and insulin-independence in type 1 diabetes from stem cell–derived, fully differentiated islet cells—Updated data from the VX-880 clinical trial. *Diabetes*. (2023) 72. doi: 10.2337/db23-836-P

25. Larkins NG, Wong G, Johnson DW, Hawley C, Teixeira-Pinto A, Pleass H, et al. Early graft loss following transplantation from expanded criteria donors. *Transplant Direct*. (2021) 7:e783. doi: 10.1097/TXD.0000000000001235

26. Fiorina P, Folli F, Maffi P, Placidi C, Venturini M, Finzi G, et al. Islet transplantation improves vascular diabetic complications in patients with diabetes who underwent kidney transplantation: a comparison between kidney-pancreas and kidney-alone transplantation. *Transplantation*. (2003) 75:1296–301. doi: 10.1097/01.TP.0000061788.32639.D9

27. Mok D, Black M, Gupta N, Arefanian H, Tredget E, Rayat GR. Early immune mechanisms of neonatal porcine islet xenograft rejection. *Xenotransplantation*. (2019) 26:e12546. doi: 10.1111/xen.12546

28. Eisenson DL, Hisadome Y, Yamada K. Progress in xenotransplantation: immunologic barriers, advances in gene editing, and successful tolerance induction strategies in pig-to-primate transplantation. *Front Immunol*. (2022) 13:899657. doi: 10.3389/fimmu.2022.899657

29. Vadori M, Cozzi E. The immunological barriers to xenotransplantation. *Tissue Antigens*. (2015) 86:239–53. doi: 10.1111/tan.12669

30. Naziruddin B, Iwahashi S, Kanak MA, Takita M, Itoh T, Levy MF. Evidence for instant blood-mediated inflammatory reaction in clinical autologous islet transplantation. *Am J Transplant*. (2014) 14:428–37. doi: 10.1111/ajt.12558

31. Nilsson B, Ekdahl KN, Korsgren O. Control of instant blood-mediated inflammatory reaction to improve islets of Langerhans engraftment. *Curr Opin Organ Transplant*. (2011) 16:620–6. doi: 10.1097/MOT.0b013e32834c2393

32. Ji M, Yi S, Smith-Hurst H, Phillips P, Wu J, Hawthorne W, et al. The importance of tissue factor expression by porcine NICC in triggering IBMIR in the xenograft setting. *Transplantation*. (2011) 91:841–6. doi: 10.1097/TP.0b013e3182106091

33. Kourtzelis I, Magnusson PU, Kotlabova K, Lambris JD, Chavakis T. Regulation of instant blood mediated inflammatory reaction (IBMIR) in pancreatic islet xeno-transplantation: points for therapeutic interventions. *Adv Exp Med Biol*. (2015) 865:171–88. doi: 10.1007/978-3-319-18603-0_11

34. Bennet W, Sundberg B, Lundgren T, Tibell A, Groth CG, Richards A, et al. Damage to porcine islets of Langerhans after exposure to human blood in *vitro*, or after intraportal transplantation to cynomologus monkeys: protective effects of sCR1 and heparin. *Transplantation*. (2000) 69:711–9. doi: 10.1097/00007890-200003150-00007

35. Miyagawa S, Yamamoto A, Matsunami K, Wang D, Takama Y, Ueno T, et al. Complement regulation in the GalT KO era. *Xenotransplantation*. (2010) 17:11–25. doi: 10.1111/xen.2010.17.issue-1

36. Davalli AM, Ogawa Y, Scaglia L, Wu YJ, Hollister J, Bonner-Weir S, et al. Function, mass, and replication of porcine and rat islets transplanted into diabetic nude mice. *Diabetes*. (1995) 44:104–11. doi: 10.2337/diab.44.1.104

37. Liu Z, Hu W, He T, Dai Y, Hara H, Bottino R, et al. Pig-to-primate islet xenotransplantation: past, present, and future. *Cell Transplant*. (2017) 26:925–47. doi: 10.3727/096368917X694859

38. Dufrane D, Goebbels RM, Gianello P. Alginate macroencapsulation of pig islets allows correction of streptozotocin-induced diabetes in primates up to 6 months without immunosuppression. *Transplantation*. (2010) 90:1054–62. doi: 10.1097/TP.0b013e3181f6e267

39. Wynyard S, Nathu D, Garkavenko O, Denner J, Elliott R. Microbiological safety of the first clinical pig islet xenotransplantation trial in New Zealand. *Xenotransplantation*. (2014) 21:309–23. doi: 10.1111/xen.12102

40. Rayat GR, Rajotte RV, Elliott JF, Korbutt GS. Expression of Gal alpha(1,3)gal on neonatal porcine islet beta-cells and susceptibility to human antibody/complement lysis. *Diabetes*. (1998) 47:1406–11. doi: 10.2337/diabetes.47.9.1406

41. Yamada K, Yazawa K, Shimizu A, Iwanaga T, Hisashi Y, Nuhn M, et al. Marked prolongation of porcine renal xenograft survival in baboons through the use of alpha1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. *Nat Med*. (2005) 11:32–4. doi: 10.1038/nm1172

42. Kuwaki K, Tseng YL, Dor FJ, Shimizu A, Houser SL, Sanderson TM, et al. Heart transplantation in baboons using alpha1,3-galactosyltransferase gene-knockout pigs as donors: initial experience. *Nat Med*. (2005) 11:29–31. doi: 10.1038/nm1171

43. Rayat GR, Rajotte RV, Hering BJ, Binette TM, Korbutt GS. *In vitro* and in *vivo* expression of Galalpha-(1,3)Gal on porcine islet cells is age dependent. *J Endocrinol*. (2003) 177:127–35. doi: 10.1677/joe.0.1770127

44. van der Windt DJ, Bottino R, Casu A, Campanile N, Smetanka C, He J, et al. Long-term controlled normoglycemia in diabetic non-human primates after transplantation with hCD46 transgenic porcine islets. *Am J Transplant*. (2009) 9:2716–26. doi: 10.1111/j.1600-6143.2009.02850.x

45. Thompson P, Badell IR, Lowe M, Cano J, Song M, Leopardi F, et al. Islet xenotransplantation using gal-deficient neonatal donors improves engraftment and function. *Am J Transplant*. (2011) 11:2593–602. doi: 10.1111/j.1600-6143.2011.03720.x

46. Hawthorne WJ, Salvaris EJ, Chew YV, Burns H, Hawkes J, Barlow H, et al. Xenotransplantation of genetically modified neonatal pig islets cures diabetes in baboons. *Front Immunol*. (2022) 13:898948. doi: 10.3389/fimmu.2022.898948

47. Yamada K, Shimizu A, Utsugi R, Ierino FL, Gargollo P, Haller GW, et al. Thymic transplantation in miniature swine. II. Induction of tolerance by transplantation of composite thymokidneys to thymectomized recipients. *J Immunol*. (2000) 164:3079–86. doi: 10.4049/jimmunol.164.6.3079

48. LaMattina JC, Kumagai N, Barth RN, Yamamoto S, Kitamura H, Moran SG, et al. Vascularized thymic lobe transplantation in miniature swine: I. Vascularized thymic lobe allografts support thymopoiesis. *Transplantation*. (2002) 73:826–31. doi: 10.1097/00007890-200203150-00032

49. Yamada K, Vagefi PA, Utsugi R, Kitamura H, Barth RN, LaMattina JC, et al. Thymic transplantation in miniature swine: III. Induction of tolerance by transplantation of composite thymokidneys across fully major histocompatibility complex-mismatched barriers. *Transplantation*. (2003) 76:530–6. doi: 10.1097/01.TP.0000080608.42480.E8

50. Kumagai N, LaMattina JC, Kamano C, Vagefi PA, Barth RN, JJ, et al. Vascularized islet cell transplantation in miniature Swine: islet-kidney allografts correct the diabetic hyperglycemia induced by total pancreatectomy. *Diabetes*. (2002) 51:3220–8. doi: 10.2337/diabetes.51.11.3220

51. Kumagai N, O'neil JJ, Barth RN, LaMattina JC, Utsugi R, Moran SG, et al. Vascularized islet-cell transplantation in miniature swine. I. Preparation of vascularized islet kidneys. *Transplantation*. (2002) 74:1223–30. doi: 10.1097/00007890-200211150-00005

52. Yamada K, Hirakata A, Tchipashvili V, Shimizu A, Iwaki H, Griesemer A, et al. Composite islet-kidneys from single baboon donors cure diabetes across fully allogenic barriers. *Am J Transplant*. (2011) 11:2603–12. doi: 10.1111/j.1600-6143.2011.03733.x

53. Pomposelli T, Wang P, Takeuchi K, Miyake K, Ariyoshi Y, Watanabe H, et al. Protection of pancreatic islets using theranostic silencing nanoparticles in a baboon model of islet transplantation. *Diabetes*. (2020) 69:2414–22. doi: 10.2337/db20-0517

54. Bottino R, Balamurugan AN, Smetanka C, Bertera S, He J, Rood PP, et al. Isolation outcome and functional characteristics of young and adult pig pancreatic islets for transplantation studies. *Xenotransplantation*. (2007) 14:74–82. doi: 10.1111/j.1399-3089.2006.00374.x

55. Kim JH, Kim HI, Lee KW, Yu JE, Kim SH, Park HS, et al. Influence of strain and age differences on the yields of porcine islet isolation: extremely high islet yields from SPF CMS miniature pigs. *Xenotransplantation*. (2007) 14:60–6. doi: 10.1111/j.1399-3089.2006.00364.x

56. Mezrich JD, Haller GW, Arn JS, Houser SL, Madsen JC, Sachs DH. Histocompatible miniature swine: an inbred large-animal model. *Transplantation*. (2003) 75:904–7. doi: 10.1097/01.TP.0000054839.43852.BF

57. Fuchimoto Y, Yamada K, Shimizu A, Yasumoto A, Sawada T, Huang CH, et al. Relationship between chimerism and tolerance in a kidney transplantation model. *J Immunol*. (1999) 162:5704–11. doi: 10.4049/jimmunol.162.10.5704.

58. Cui W, Gu Y, Miyamoto M, Tanaka M, Xu B, Imamura M, et al. Novel method for isolation of adult porcine pancreatic islets with two-stage digestion procedure. *Cell Transplant*. (1999) 8:391–8. doi: 10.1177/096368979900800408

59. Griesemer AD, Lamattina JC, Okumi M, Etter JD, Shimizu A, Sachs DH, et al. Linked suppression across an MHC-mismatched barrier in a miniature swine kidney transplantation model. *J Immunol*. (2008) 181:4027–36. doi: 10.4049/jimmunol.181.6.4027

frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 22 March 2024
DOI 10.3389/fimmu.2024.1386382

OPEN ACCESS

EDITED BY
Lisha Mou,
Shenzhen Second People's Hospital, China

REVIEWED BY
Dafei Chai,
Xuzhou Medical University, China
Rongkai Yan,
The Ohio State University, United States

*CORRESPONDENCE
Yi Wang
w_yi2022@163.com
Jianzhen Lv
cancan912@163.com;
Si-Yuan Song
si-yuan.song@bcm.edu

†These authors have contributed equally to this work

RECEIVED 15 February 2024
ACCEPTED 13 March 2024
PUBLISHED 22 March 2024

CITATION
Zhang X, Wang H, Xie Q, Zhang Y, Yang Y, Yuan M, Cui Y, Song S-Y, Lv J and Wang Y (2024) Advancing kidney xenotransplantation with anesthesia and surgery - bridging preclinical and clinical frontiers challenges and prospects.
Front. Immunol. 15:1386382.
doi: 10.3389/fimmu.2024.1386382

Advancing kidney xenotransplantation with anesthesia and surgery - bridging preclinical and clinical frontiers challenges and prospects

Xiaojian Zhang[1†], Hailian Wang[2†], Qin Xie[3†], Yang Zhang[4†], Yixin Yang[5], Man Yuan[6], Yuqi Cui[7], Si-Yuan Song[8*], Jianzhen Lv[9*] and Yi Wang[2*]

[1]Central of Reproductive Medicine, Department of Obstetrics and Gynecology, Sichuan Academy of Medical Science and Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China, Chengdu, China, [2]Clinical Immunology Translational Medicine Key Laboratory of Sichuan Province, Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China, Chengdu, China, [3]Department of Critical Care Medicine, Zhongnan Hospital of Wuhan University, Wuhan, China, [4]Department of Anesthesiology, Perioperative and Pain Medicine, Stanford University School of Medicine, Standford, CA, United States, [5]The First Clinical Medical College of Norman Bethune University of Medical Sciences, Jilin, China, [6]Eye Center, Renmin Hospital of Wuhan University, Wuhan University, Wuhan, Hubei, China, [7]Department of Geriatrics, Donald W. Reynolds Institute on Aging, University of Arkansas for Medical Sciences, Little Rock, AR, United States, [8]Department of Neuroscience, Baylor College of Medicine, Houston, TX, United States, [9]School of Pharmacy, Guangxi University of Chinese Medicine, Nanning, China

Xenotransplantation is emerging as a vital solution to the critical shortage of organs available for transplantation, significantly propelled by advancements in genetic engineering and the development of sophisticated immunosuppressive treatments. Specifically, the transplantation of kidneys from genetically engineered pigs into human patients has made significant progress, offering a potential clinical solution to the shortage of human kidney supply. Recent trials involving the transplantation of these modified porcine kidneys into deceased human bodies have underscored the practicality of this approach, advancing the field towards potential clinical applications. However, numerous challenges remain, especially in the domains of identifying suitable donor-recipient matches and formulating effective immunosuppressive protocols crucial for transplant success. Critical to advancing xenotransplantation into clinical settings are the nuanced considerations of anesthesia and surgical practices required for these complex procedures. The precise genetic modification of porcine kidneys marks a significant leap in addressing the biological and immunological hurdles that have traditionally challenged xenotransplantation. Yet, the success of these transplants hinges on the process of meticulously matching these organs with human recipients, which demands thorough understanding of immunological compatibility, the risk of organ rejection, and the prevention of zoonotic disease transmission. In parallel, the development and optimization of immunosuppressive protocols are imperative to mitigate rejection risks while minimizing side effects, necessitating innovative approaches in both pharmacology and clinical practices. Furthermore, the

post-operative care of recipients, encompassing vigilant monitoring for signs of organ rejection, infectious disease surveillance, and psychological support, is crucial for ensuring post-transplant life quality. This comprehensive care highlights the importance of a multidisciplinary approach involving transplant surgeons, anesthesiologists, immunologists, infectiologists and psychiatrists. The integration of anesthesia and surgical expertise is particularly vital, ensuring the best possible outcomes of those patients undergoing these novel transplants, through safe procedural practices. As xenotransplantation moving closer to clinical reality, establishing consensus guidelines on various aspects, including donor-recipient selection, immunosuppression, as well as surgical and anesthetic management of these transplants, is essential. Addressing these challenges through rigorous research and collective collaboration will be the key, not only to navigate the ethical, medical, and logistical complexities of introducing kidney xenotransplantation into mainstream clinical practice, but also itself marks a new era in organ transplantation.

KEYWORDS

xenogeneic kidney transplantation, genetically modified pigs, anesthesia, immunological compatibility, organ rejection, immunotherapy, surgical techniques

1 Introduction

Kidney transplantation is thought of the gold standard treatment for those end-stage renal disease patients, which will present substantial improvement in quality of life and life expectancy. This procedure, particularly allogenic kidney transplantation, has achieved remarkable success in extending lives (1, 2). Nonetheless, the ongoing shortage of available organs for transplantation represents a significant hurdle, failing clinical demand. In response, the medical community has broadened the donor pool, such as utilizing organs from both deceased and living donors, including those marginal donors or undergoing ABO-incompatible transplants. Such efforts, while commendable, have not sufficiently mitigated the organ scarcity crisis. This situation underscores the urgent necessity for innovative strategies to augment the organ donor pool. Integrating advanced surgical techniques and anesthesia practices in the transplantation process is also vital, not only to ensure patient safety during these complex procedures but also to potentially increase the viability of organs from the marginal donors for transplantation. By refining surgical and anesthetic methodologies, the transplantation community can enhance post-operative outcomes and expand the criteria for donor acceptance, thereby addressing the pressing demand for kidney transplants (1).

Xenotransplantation, the process of transplanting organs or tissues between different species, has emerged as a pivotal solution in the quest to alleviate the organ shortage. Recent advancements in the realms of genetic engineering and immunosuppression, particularly through the use of targeted monoclonal antibodies, have propelled forward the research into kidney xenotransplantation. Experiments involving the transplantation of genetically modified porcine kidneys into non-human recipients have demonstrated significant progress, through those extended graft survival times reported (3). These achievements underscore the potential of xenotransplantation to bridge the gap in organ demand. However, translating these findings to human recipients is met with caution, due to the inherent biological and immunological differences, as well as the looming concerns over safety and ethical implications. The journey toward the clinical application of porcine-to-human kidney transplants is fraught with uncertainties, necessitating rigorous investigation and ethical deliberation.

The exploration of xenotransplantation, as a viable source of kidneys for transplantation, is gaining momentum, underpinned by the pressing need to address the organ shortage crisis. This approach not only promises to expand the donor pool but also to pioneer new frontiers in transplantation medicine (4). Advances in genetic modification techniques aim to enhance organ compatibility and reduce the likelihood of rejection and cross-species infection, marking a significant leap towards making xenotransplantation a reality for patients in need (4).

Moreover, the field of xenotransplantation is evolving alongside considerations of ethical standards and regulatory frameworks, ensuring that the development and potential clinical implementation are conducted with the utmost care for safety and ethical integrity. As the scientific community continues to unravel the complexities of xenogeneic organ transplantation, the collective goal remains clear: to provide a sustainable, ethical solution to the organ shortage crisis, ultimately saving more lives through innovative medical breakthroughs (5).

In essence, the path forward in xenotransplantation research and its prospective clinical application is laden with challenges yet brimming with potential. Through continued interdisciplinary collaboration, rigorous ethical scrutiny, and innovative scientific inquiry, xenotransplantation holds the promise of significantly impacting the future of organ transplantation, offering hope to countless individuals awaiting life-saving transplants.

2 Donor selection in kidney transplantation

In the advancement of xenogeneic kidney transplantation, the selection and genetic engineering of donor animals are paramount to ensuring the safety and efficacy of this innovative treatment approach. Due to the potential risk of virus transmission that makes non-human primates (NHPs) less suitable as xenotransplant donors, pigs have become the preferred source for donor organs. Pig kidneys, with their physiological and anatomical similarities to human kidneys, offer a practical alternative. They not only closely match human kidneys in size and function but also have the advantage of rapid reproduction and the capability for precise genetic modifications. However, the considerable genetic disparities between pigs and humans pose a significant challenge, often resulting in strong immune rejection and the failure of the transplant. To counteract these issues, genetic engineering plays a critical role.

The advent of CRISPR/Cas9 gene-editing technology has been a game-changer in this field. By targeting specific genes, scientists can reduce the risk of hyperacute and acute rejection responses. For instance, the elimination of the α-1,3-galactosyltransferase (GGTA1) gene in pigs prevents the expression of the αGal xenoantigen, significantly lowering the chances of hyperacute rejection. Further modifications, such as the deletion of the β-1,4-N-acetylgalactosaminyltransferase (β4GalNT2) and cytidine monophospho-N-acetylneuraminic acid hydroxylase (CMAH) genes, have been shown to mitigate acute vascular rejections that are not mediated by αGal antigens (6). The integration of human genes encoding complement regulatory proteins, anticoagulants, immune regulators, and other protective elements into the pig genome further enhances the compatibility of porcine kidneys with human recipients. These include genes for human complement regulatory proteins (hCD46 and hCD55), which help protect the transplanted organ from the recipient's immune system, and genes like human thrombomodulin (hTBM) and human endothelial protein C receptor (hEPCR), which work to prevent clotting and improve graft survival (6–11) (Figure 1).

Research institutions, such as the University of Alabama, have made significant strides by utilizing pigs with a comprehensive suite of genetic modifications, including enhancements to immune regulation and coagulation profiles, as well as the removal of specific antigens known to trigger rejection (12). This meticulous genetic engineering aims to produce organs that are not only less likely to be rejected but also capable of performing their physiological functions without inducing harmful side effects in the recipient.

The continuous exploration and refinement of these genetic modifications underscore the complexity of making xenotransplantation a viable clinical option. As researchers dive deeper into the genetic underpinnings of immune rejection and organ compatibility, xenotransplantation alleviating the organ shortage crisis is coming true. Yet, it is essential to balance the enthusiasm for these technological advances with a careful assessment of their long-term implications for both organ recipients and the broader field of transplantation medicine.

Future research must also address the ethical considerations and regulatory challenges associated with xenotransplantation. As genetic engineering techniques become more sophisticated, consensus of the moral, ethical, and societal implications of using genetically modified animals for organ transplantation must be reached. Those topics warranting immediate attention includes animal welfare, the potential impact on natural ecosystems, and the long-term health outcomes for transplant recipients. Moreover, as the field progresses towards potential clinical applications, establishing clear and comprehensive regulatory frameworks will be crucial to ensuring the safety, efficacy, and ethical integrity of organ xenotransplantation.

While the genetic engineering of pigs for kidney xenotransplantation represents a frontier of medical science with the potential to dramatically expand the organ donor pool, it also poses a myriad of scientific, ethical, and regulatory challenges. Navigating these complexities will require concerted efforts from researchers, ethicists, and policymakers, with the ultimate goal of making xenotransplantation a safe, ethical, and effective solution for the millions worldwide in need of life-saving organ transplants.

3 Xenotransplantation recipient selection

Historically, xenotransplantation has traversed a challenging path, with initial attempts to transplant animal organs into humans dating back to the early 20th century, notably beginning in 1906 (13). The pursuit of understanding xenotransplantation's complexities led researchers to utilize animal models that have smaller body sizes, despite their significant genetic differences from humans (e.g., a 33.4% nucleotide difference between mice and rats, compared to a 2.6% difference between macaques and baboons). These models, particularly rodents, were instrumental in elucidating the humoral and cellular dynamics of xenotransplantation. Up until 2012, rodents were the backbone of xenotransplantation studies, contributing to approximately 95% of research in the field. The advent of sophisticated genetic editing technologies has recently shifted the preference towards NHPs as models, given their closer genetic proximity to humans, which allows for more accurate simulation of immunosuppressive strategies and rejection mechanisms.

The utilization of brain-dead individuals as recipients of pig kidney transplants has served as a crucial intermediary step towards clinical xenotransplantation research (14). Although this model has provided invaluable insights, it is limited by the short survival times

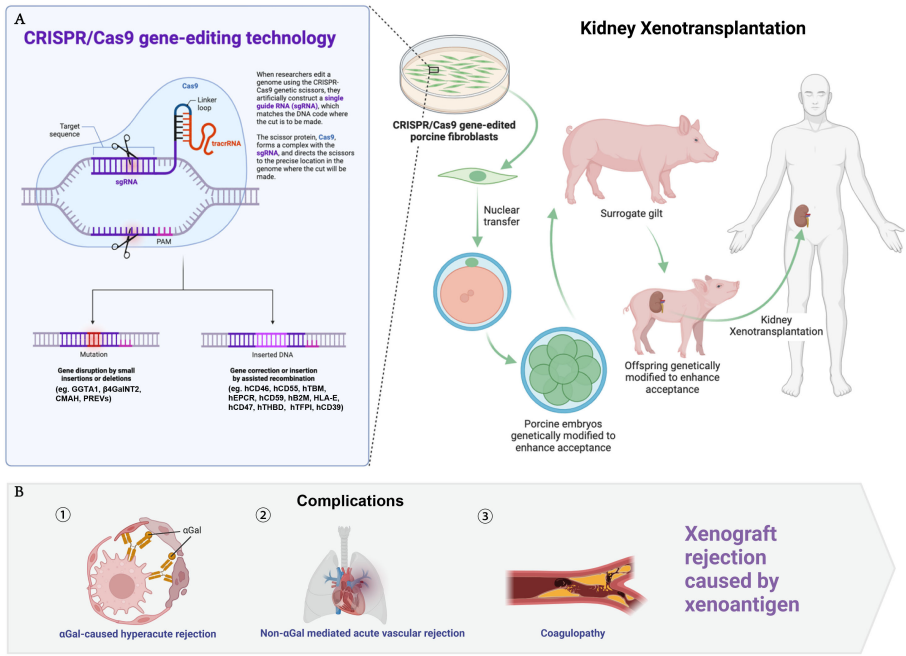


FIGURE 1
Genetic modifications in donor pigs for kidney xenotransplantation. This figure illustrates the CRISPR/Cas9 gene-editing technology and its application to donor pigs to enhance compatibility for kidney xenotransplantation **(A)**, while part B addresses the complications related to xenograft rejection **(B)**. The genetic modifications are listed as follows: GGTA1, alpha-1,3-galactosyltransferase; β4GalNT2, beta-1,4-N-acetylgalactosaminyltransferase 2; CMAH, cytidine monophosphate-N-acetylneuraminic acid hydroxylase; PREVs, porcine endogenous retroviruses, including PERV-A, PERV-B, PERV-C, inactivated to prevent cross-species viral transmission; hCD46, human membrane cofactor protein; hCD55, human decay-accelerating factor; hTBM, human thrombomodulin; hEPCR, human endothelial protein C receptor; hCD59, human protectin; hB2M, human beta-2 microglobulin; HLA-E, human leukocyte antigen E; hCD47, human integrin-associated protein; hTHBD, human thrombomodulin; hTFPI, human tissue factor pathway inhibitor; hCD39, human ectonucleoside triphosphate diphosphohydrolase 1.

of the recipients, which restricts the exploration of long-term complications such as viral transmissions or abnormal organ growth (15). Establishing a standardized selection criterion for human recipients of xenotransplant organs remains a work in progress. Proposals suggest prioritizing individuals for whom conventional treatments have failed, or who face long waiting time for allogeneic transplants due to immunological sensitivities, such as a high level of panel reactive antibodies (PRA) (16). However, the selection of highly sensitized patients poses its own set of challenges, including the potential for adverse reactions between pig (swine leukocyte antigen, SLA) and human (human leukocyte antigen, HLA) antigens (16, 17). The field of xenotransplantation is at a critical juncture, requiring expanded research efforts to explore not only the scientific and ethical ramifications of these procedures but also the practical aspects of preparing for and managing post-transplant care. While the literature is currently sparse on the preoperative preparation and rehabilitation of recipients in the context of xenotransplantation, the lessons learned from allogeneic transplantation underscore the importance of comprehensive preoperative assessments and perioperative care. The clinical trials conducted at New York University and the University of Alabama at Birmingham involving xenotransplantation of kidneys into brain-dead human subjects have yielded valuable insights for perioperative management. Standard protocols for managing brain-dead patients before organ donation typically involve the administration of vasopressors, levothyroxine, steroids, and other interventions aimed at maintaining normal hemodynamics. During intraoperative anesthesia care, efforts mirror those in human-to-human kidney transplantation, encompassing administering immunosuppressive agents, maintaining metabolic stability, and optimizing hemodynamics to ensure adequate renal perfusion. To translate these findings into clinical practice, adherence to established standards of care is imperative. These pilot studies primarily addressed early-phase recovery, focusing on aspects such as hyperacute rejection, intraoperative life-threatening complications, and kidney function. Their findings lay the groundwork for future investigations to refine clinical practices in xenotransplantation (12, 18).. As xenotransplantation inches closer to clinical reality, the focus must also broaden to include patient rehabilitation and long-term care strategies that are tailored to the unique challenges of xenogeneic organ transplants. Additionally, the exploration of xenotransplantation as a bridge therapy for patients with end-stage renal disease presents an area ripe for investigation. This approach could potentially offer a lifeline to patients awaiting allogeneic transplants, with the added advantage of not precluding future allogeneic kidney transplants (19). The integration of xenotransplantation into the broader organ transplantation field raises complex questions regarding organ functionality, ethical considerations, and long-term patient outcomes. With kidneys playing a multifaceted role in human physiology, determining whether pig kidneys can fully meet human needs requires meticulous study. As research progresses, it will be crucial to

develop clear guidelines for recipient selection, manage expectations regarding the outcomes of xenotransplantation, and ensure ethical standards are upheld in the pursuit of expanding the organ donor pool.

4 Challenges in kidney xenotransplantation

Xenotransplantation presents a multifaceted array of challenges, from immunological barriers to ethical considerations, each requiring meticulous attention to ensure the viability and success of organ transplants from pigs to humans.

The immunosuppressive regimen is pivotal in xenotransplantation, with a focus on minimizing the recipient's immune response to the xenograft. Standard immunosuppressants such as tacrolimus, cyclosporine, mycophenolic acid (MPA), sirolimus, and corticosteroids form the cornerstone of current strategies. The evolution of immunosuppression has seen the introduction of novel agents targeting specific pathways critical for T-cell activation and the complement system. Anti-CD40 and anti-CD154 antibodies, for instance, have demonstrated potential in prolonging the survival of xenografts by inhibiting the CD40/CD154 co-stimulation pathway, a crucial step in T-cell mediated rejection (20, 21). Meanwhile, complement system inhibition, necessary to avert hyperacute rejection and thrombosis, relies on advanced strategies such as the use of C1 inhibitors and monoclonal antibodies like sutimlimab to suppress complement activation (22–24).

The physiological functions of pig kidneys, including erythropoietin (EPO) production and the regulation of the renin-angiotensin-aldosterone system (RAAS), along with maintaining proper acid-base balance, are critical for the graft's integration and function within the human body (25, 26). The brain-dead decedents experience disrupted homeostasis and physiological functions, posing significant challenges even in critical care settings to preserve hemodynamic, hormonal, metabolic, and immune stability. In the initial pilot studies, standard intraoperative anesthesia and postoperative critical care protocols were implemented to ensure the maintenance of these vital parameters, mirroring established clinical practices (12, 18). The common complications include coagulopathy, stemming from endothelial damage and acute rejection, further complicate the post-transplant scenario, necessitating ongoing research and development of strategies to mitigate these effects.

Viral infections, particularly those associated with porcine endogenous retroviruses (PERV) and porcine cytomegalovirus (PCMV/PRV), pose significant risks for xenotransplantation. Nevertheless, both preclinical and clinical investigations conducted thorough pathogen screening to exclude prevalent viruses in pig donors, such as PERV-3 and PCMV. The post-transplantation virus detection in the decedents also remained negative (12, 27) However, the limitations of these negative findings are underscored by the relatively short observation periods. Consequently, developing future strategies to detect and eliminate these viruses is imperative to uphold the safety of both the graft and the recipient (28).

Additionally, the ethical landscape of xenotransplantation, encompassing animal rights, public attitudes, and regulatory milestones such as the FDA's 2022 approval of a pig-to-human heart transplant, presents ongoing challenges (29). The pilot xenotransplantation trials have underscored various ethical and medicolegal considerations inherent in xenotransplantation research (12, 18). In these studies, brain-dead decedents were precluded from organ donation, and obtaining proper informed consent from their families was deemed essential. Notably, the absence of specific legislation governing xenotransplantation necessitates evolution of the regulatory frameworks to enhance research protocols and future clinical applications. Consultation with ethics committees is imperative to ensure adherence to established guidelines such as the Uniform Anatomical Gift Act (UAGA) and the dead-donor rule, while also acknowledging the cultural and religious nuances surrounding organ transplantation (30). Public acceptance and ethical considerations remain integral to the advancement and clinical application of xenotransplantation, highlighting the need for continued research, dialogue, and education in this evolving field.

In summary, xenotransplantation's journey towards becoming a viable clinical option is fraught with complex immunological, physiological, virological, and ethical challenges. Each step forward requires a careful balance of innovation, safety, and ethical considerations, with the ultimate goal of expanding the organ donor pool and saving lives.

5 Conclusion and perspectives

Xenotransplantation has shown considerable promise in early-stage studies, bridging a critical gap between theoretical potential and practical clinical application (31). This transition from laboratory success to real-world efficacy underscores the importance of preclinical research as an essential step. This phase serves not only to validate findings from controlled laboratory settings in more clinically relevant scenarios but also to identify unforeseen challenges that may not be apparent in initial studies. The intricate dance between clinical application and laboratory research is informed by these challenges, directing the trajectory of future investigations. The exploration of genetically engineered pigs, particularly those modified with multiple genes, marks a significant advancement in this field (3). However, delving deeper into the specific functions of these genetic modifications and the discovery of new xenoantigens remain critical areas for further research. The protracted process of breeding these genetically altered pigs also poses a logistical challenge, emphasizing the need for streamlining breeding techniques to enhance research efficiency.

The ethical considerations surrounding the selection of participants for clinical trials, especially the inclusion of end-stage renal disease patients or those not eligible for conventional transplants, continue to provoke debate. Alternatively, the use of brain-dead individuals in preclinical studies presents a less contentious pathway, aligning with both ethical standards and research needs. Meanwhile, the quest for optimal immunosuppression strategies remains ongoing, with the current regimens requiring refinement to improve outcomes and reduce adverse effects. The consistency in selecting donor

genotypes and standardizing perioperative care protocols also presents a significant hurdle, mirroring the complexity of translating xenotransplantation into a clinically viable option. Persistent issues such as graft rejection, inflammation, coagulation disorders, maintaining the physiological function of transplanted kidneys, and managing the risk of viral transmission underscore the multifaceted challenges ahead.

As preclinical research progresses, it is imperative to tackle these obstacles head-on, paving the way for the successful integration of xenotransplantation into clinical practice. The future of this innovative field hinges on our ability to navigate these complexities, requiring a concerted effort from researchers, clinicians, and ethicists alike. By addressing the nuanced challenges of genetic engineering, immunosuppression, and clinical trial design, xenotransplantation can move closer to becoming a tangible solution for the organ shortage crisis. Furthermore, enhancing the understanding of xenograft physiology and immunology will be crucial in developing targeted interventions that minimize rejection and improve long-term graft survival. Through these endeavors, xenotransplantation stands on the cusp of transitioning from an experimental procedure to a revolutionary treatment modality, offering hope to thousands of patients awaiting life-saving organ transplants.

Author contributions

XZ: Writing – original draft. SS: Writing – original draft. HW: Writing – original draft. QX: Writing – original draft. YZ: Writing – original draft. YY: Writing – original draft. MY: Writing – original draft. YC: Writing – original draft. JL: Funding acquisition, Writing – review & editing. YW: Conceptualization, Funding acquisition, Investigation, Project administration, Validation, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This research was supported by the National Natural Science Foundation of China (81802504), grants from the Sichuan Science and Technology Bureau (20211YFH0384 and 2023YFH0010), and a grant from Guangxi Natural Science Foundation (2022GXNSFAA035608).

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.

Publisher's note

References

1. Zhou Q, Li T, Wang K, Zhang Q, Geng Z, Deng S, et al. Current status of xenotransplantation research and the strategies for preventing xenograft rejection. *Front Immunol.* (2022) 13:928173. doi: 10.3389/fimmu.2022.928173

2. Hariharan S, Israni AK, Danovitch G. Long-term survival after kidney transplantation. *N Engl J Med.* (2021) 385:729–43. doi: 10.1056/NEJMra2014530

3. Anand RP, Layer JV, Heja D, Hirose T, Lassiter G, Firl DJ, et al. Design and testing of a humanized porcine donor for xenotransplantation. *Nature.* (2023) 622:393–401. doi: 10.1038/s41586-023-06594-4

4. Cooper DKC, Hara H, Iwase H, Yamamoto T, Jagdale A, Kumar V, et al. Clinical pig kidney xenotransplantation: how close are we? *J Am Soc Nephrol.* (2020) 31:12–21. doi: 10.1681/ASN.2019070651

5. Cooper DKC, Hara H, Iwase H, Yamamoto T, Wang ZY, Jagdale A, et al. Pig kidney xenotransplantation: Progress toward clinical trials. *Clin Transplant.* (2021) 35: e14139. doi: 10.1111/ctr.14139

6. Tector AJ, Mosser M, Tector M, Bach JM. The possible role of anti-neu5Gc as an obstacle in xenotransplantation. *Front Immunol.* (2020) 11:622. doi: 10.3389/fimmu.2020.00622

7. Cooper DKC, Hara H, Iwase H, Yamamoto T, Li Q, Ezzelarab M, et al. Justification of specific genetic modifications in pigs for clinical organ xenotransplantation. *Xenotransplantation.* (2019) 26:e12516. doi: 10.1111/xen.12516

8. Nagano F, Mizuno T, Mizumoto S, Yoshioka K, Takahashi K, Tsuboi N, et al. Chondroitin sulfate protects vascular endothelial cells from toxicities of extracellular histones. *Eur J Pharmacol.* (2018) 826:48–55. doi: 10.1016/j.ejphar.2018.02.043

9. Watanabe H, Ariyoshi Y, Pomposelli T, Takeuchi K, Ekanayake-Alper DK, Boyd LK, et al. Intra-bone bone marrow transplantation from hCD47 transgenic pigs to baboons prolongs chimerism to >60 days and promotes increased porcine lung transplant survival. *Xenotransplantation.* (2020) 27:e12552. doi: 10.1111/xen.12552

10. Niu D, Wei HJ, Lin L, George H, Wang T, Lee IH, et al. Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. *Science.* (2017) 357:1303–7. doi: 10.1126/science.aan4187

11. Yue Y, Xu W, Kan Y, Zhao HY, Zhou Y, Song X, et al. Extensive germline genome engineering in pigs. *Nat BioMed Eng.* (2021) 5:134–43. doi: 10.1038/s41551-020-00613-9

12. Porrett PM, Orandi BJ, Kumar V, Houp J, Anderson D, Cozette Killian A, et al. First clinical-grade porcine kidney xenotransplant using a human decedent model. *Am J Transplant.* (2022) 22:1037–53. doi: 10.1111/ajt.16930

13. Siems C, Huddleston S, John R. A brief history of xenotransplantation. *Ann Thorac Surg.* (2022) 113:706–10. doi: 10.1016/j.athoracsur.2022.01.005

14. Bayliss G. Practical ethical concerns in allocation of pig kidneys to humans. *Clin Kidney J.* (2022) 15:2161–8. doi: 10.1093/ckj/sfac125

15. Ganchiku Y, Riella LV. Pig-to-human kidney transplantation using brain-dead donors as recipients: One giant leap, or only one small step for transplantkind? *Xenotransplantation.* (2022) 29:e12748. doi: 10.1111/xen.12748

16. Yu XH, Deng WY, Jiang HT, Li T, Wang Y. Kidney xenotransplantation: Recent progress in preclinical research. *Clin Chim Acta.* (2021) 514:15–23. doi: 10.1016/j.cca.2020.11.028

17. Ladowski JM, Hara H, Cooper DKC. The role of SLAs in xenotransplantation. *Transplantation*. (2021) 105:300–7. doi: 10.1097/TP.0000000000003303

18. Montgomery RA, Stern JM, Lonze BE, Tatapudi VS, Mangiola M, Wu M, et al. Results of two cases of pig-to-human kidney xenotransplantation. *N Engl J Med*. (2022) 386:1889–98. doi: 10.1056/NEJMoa2120238

19. Hara H, Nguyen H, Wang ZY, Jagdale A, Bikhet M, Yamamoto T, et al. Evidence that sensitization to triple-knockout pig cells will not be detrimental to subsequent allotransplantation. *Xenotransplantation*. (2021) 28:e12701. doi: 10.1111/xen.12701

20. Mohiuddin MM, Singh AK, Corcoran PC, Thomas Iii ML, Clark T, Lewis BG, et al. Chimeric 2C10R4 anti-CD40 antibody therapy is critical for long-term survival of GTKO.hCD46.hTBM pig-to-primate cardiac xenograft. *Nat Commun*. (2016) 7:11138. doi: 10.1038/ncomms11138

21. Adams AB, Kim SC, Martens GR, Ladowski JM, Estrada JL, Reyes LM, et al. Xenoantigen deletion and chemical immunosuppression can prolong renal xenograft survival. *Ann Surg*. (2018) 268:564–73. doi: 10.1097/SLA.0000000000002977

22. Viglietti D, Gosset C, Loupy A, Deville L, Verine J, Zeevi A, et al. C1 inhibitor in acute antibody-mediated rejection nonresponsive to conventional therapy in kidney transplant recipients: A pilot study. *Am J Transplant*. (2016) 16:1596–603. doi: 10.1111/ajt.13663

23. Roth A, Barcellini W, D'Sa S, Miyakawa Y, Broome CM, Michel M, et al. Sutimlimab in cold agglutinin disease. *N Engl J Med*. (2021) 384:1323–34. doi: 10.1056/NEJMoa2027760

24. Eskandary F, Jilma B, Muhlbacher J, Wahrmann M, Regele H, Kozakowski N, et al. Anti-C1s monoclonal antibody BIVV009 in late antibody-mediated kidney allograft rejection-results from a first-in-patient phase 1 trial. *Am J Transplant*. (2018) 18:916–26. doi: 10.1111/ajt.14528

25. Hansen-Estruch C, Cooper DKC, Judd E. Physiological aspects of pig kidney xenotransplantation and implications for management following transplant. *Xenotransplantation*. (2022) 29:e12743. doi: 10.1111/xen.12743

26. Hansen-Estruch C, Bikhet MH, Javed M, Katsurada A, Satou R, Shao W, et al. Renin-angiotensin-aldosterone system function in the pig-to-baboon kidney xenotransplantation model. *Am J Transplant*. (2023) 23:353–65. doi: 10.1016/j.ajt.2022.11.022

27. Denner J. Why was PERV not transmitted during preclinical and clinical xenotransplantation trials and after inoculation of animals? *Retrovirology*. (2018) 15:28. doi: 10.1186/s12977-018-0411-8

28. Denner J. Porcine endogenous retroviruses and xenotransplantation, 2021. *Viruses*. (2021) 13(11):2156. doi: 10.3390/v13112156

29. Singh AK, Griffith BP, Goerlich CE, Ayares D, Mohiuddin MM. The road to the first FDA-approved genetically engineered pig heart transplantation into human. *Xenotransplantation*. (2022) 29:e12776. doi: 10.1111/xen.12776

30. Cooper DKC, Kobayashi T. Xenotransplantation experiments in brain-dead human subjects-A critical appraisal. *Am J Transplant*. (2023) 28:S1600-35(23)00923-1. doi: 10.1016/j.ajt.2023.12.020

31. Lunney JK, Van Goor A, Walker KE, Hailstock T, Franklin J, Dai C. Importance of the pig as a human biomedical model. *Sci Transl Med*. (2021) 13:eabd5758. doi: 10.1126/scitranslmed.abd5758

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 26 March 2024
DOI 10.3389/fimmu.2024.1379742

OPEN ACCESS

EDITED BY
Lisha Mou,
Shenzhen Second People's Hospital, China

REVIEWED BY
Chong Dong,
Tianjin First Central Hospital, China
Xin Hu,
National Center for Child Health and Development (NCCHD), Japan

*CORRESPONDENCE
Yi Sun
sunyimao123@hotmail.com
Xin-Li Mao
maoxl@enzemed.com
Shao-Wei Li
li_shaowei81@hotmail.com

†These authors have contributed equally to this work

RECEIVED 31 January 2024
ACCEPTED 14 March 2024
PUBLISHED 26 March 2024

CITATION
Huang C-L, Fu X-Y, Feng Y, Li X-K, Sun Y, Mao X-L and Li S-W (2024) Relationship between the microenvironment and survival in kidney transplantation: a bibliometric analysis from 2013 to 2023.
Front. Immunol. 15:1379742.
doi: 10.3389/fimmu.2024.1379742

Relationship between the microenvironment and survival in kidney transplantation: a bibliometric analysis from 2013 to 2023

Chun-Lian Huang[1†], Xin-Yu Fu[2†], Yi Feng[2], Xiao-Kang Li[3,4], Yi Sun[5*], Xin-Li Mao[3,6,7*] and Shao-Wei Li[3,6,7*]

[1]Department of Infectious Diseases, Taizhou Hospital of Zhejiang Province Affiliated to Wenzhou Medical University, Linhai, Zhejiang, China, [2]Taizhou Hospital of Zhejiang Province affiliated to Wenzhou Medical University, Linhai, Zhejiang, China, [3]Key Laboratory of Minimally Invasive Techniques and Rapid Rehabilitation of Digestive System Tumor of Zhejiang Province, Linhai, Zhejiang, China, [4]Division of Transplantation Immunology, National Research Institute for Child Health and Development, Tokyo, Japan, [5]MRL Global Medical Affairs, MSD China, Shanghai, China, [6]Department of Gastroenterology, Taizhou Hospital of Zhejiang Province affiliated to Wenzhou Medical University, Linhai, Zhejiang, China, [7]Institute of Digestive Disease, Taizhou Hospital of Zhejiang Province Affiliated to Wenzhou Medical University, Linhai, China

Background: Kidney transplantation is considered the most effective treatment for end-stage renal failure. Recent studies have shown that the significance of the immune microenvironment after kidney transplantation in determining prognosis of patients. Therefore, this study aimed to conduct a bibliometric analysis to provide an overview of the knowledge structure and research trends regarding the immune microenvironment and survival in kidney transplantation.

Methods: Our search included relevant publications from 2013 to 2023 retrieved from the Web of Science core repository and finally included 865 articles. To perform the bibliometric analysis, we utilized tools such as VOSviewer, CiteSpace, and the R package "bibliometrix". The analysis focused on various aspects, including country, author, year, topic, reference, and keyword clustering.

Results: Based on the inclusion criteria, a total of 865 articles were found, with a trend of steady increase. China and the United States were the countries with the most publications. Nanjing Medical University was the most productive institution. High-frequency keywords were clustered into 6 areas, including kidney transplantation, transforming growth factor β, macrophage, antibody-mediated rejection, necrosis factor alpha, and dysfunction. Antibody mediated rejection (2019-2023) was the main area of research in recent years.

Conclusion: This groundbreaking bibliometric study comprehensively summarizes the research trends and advances related to the immune microenvironment and survival after kidney transplantation. It identifies recent frontiers of research and highlights promising directions for future studies, potentially offering fresh perspectives to scholars in the field.

KEYWORDS

CiteSpace, bibliometrics, VOSview, kidney transplantation, immune microenvironment, survival

1 Introduction

Kidney transplantation has become the preferred procedure for the treatment of patients with kidney failure because it is the most effective treatment, both medically and economically (1–3). Advances in immunosuppressive drugs and protocols have markedly reduced the incidence of graft rejection and improved survival rates of patients in recent years (3–5). Nonetheless, improving long-term transplant outcomes remains a crucial challenge (4). Many current studies have shown that allograft reaction is the major cause of late kidney transplant failure (6–8). Therefore, new treatments are necessary to improve long-term graft survival and suppress allograft reactions.

Many studies have identified that the immune microenvironment (immune cells, cytokines, etc.) plays a key role in coordinating the immune response after kidney transplantation. Therefore, they can be investigated for potential applications in new therapeutic strategies (9–11). Cells in the immune microenvironment play a critical role in prolonging the survival of kidney transplant patients after kidney transplantation (12, 13). Therefore, this study systematically explores the publications and hotspots of research related to the relationship between the immune microenvironment and patient survival after kidney transplantation.

The term "bibliometric analysis" refers to the use of mathematical and statistical methods that are commonly used to provide a comprehensive picture of the current status of a field, publication trends, scientific output of researchers, institutions, and countries, and future research hotspots (14, 15). This method has been widely used in several fields. To the best of our knowledge, there have been no published bibliometric analyses of the immune microenvironment after kidney transplantation. Therefore, this study is aimed to reveal difficult problems and research hotspots related to the immune microenvironment after kidney transplantation over the past 10 years.

2 Materials and methods

2.1 Data collection

We conducted a literature search on the Web of Science Core Collection (WoSCC) database on August 21. The search formula was as follow TS = ("Cytokines" OR "Chemokines" OR "Growth Differentiation Factor 15" OR "Hematopoietic Cell Growth Factors" OR "Hepatocyte Growth Factor" OR "Interferons" OR "Interleukin 1 Receptor Antagonist Protein" OR "Interleukins" OR "Leukemia Inhibitory Factor" OR "Lymphokines" OR "Monokines" OR "Oncostatin M" OR "Osteopontin" OR "Thymic Stromal Lymphopoietin" OR "Transforming Growth Factor beta" OR "Tumor Necrosis Factors") AND ("Kidney Transplantation") AND ("Language = English"), and the type of documents is set to "articles". The search was limited to the period from August 21, 2013 to August 21, 2024. Two authors read the abstract and full text and exclude articles that are not relevant to the articles. The flow chart of the included articles is shown in Figure 1, and a total of 865 articles were selected for bibliometric analysis.

2.2 Data analysis

In this study, CiteSpace 6.1. R3, VOSviewer 1.6.18, and Microsoft Excel 2019 were used for the bibliometric analysis, visualization methods, and integration analysis (5, 16). VOSviewer can extract key information from a wide range of publications, including lead authors, and analyze country and institution, keywords, scientific partnerships, citations, and co-citations (17). CiteSpace explores the current state of research, research hotspots, research frontiers, and development process of a scientific field by generating a series of visual knowledge maps that reveal the trends in the field (18). The Excel software program was used to analyze the annual publications.

3 Results

3.1 Annual publications

Based on our inclusion criteria (Figure 1), a total of 865 articles were included in the study. As shown in Figure 2, the number of papers related to the immune microenvironment after kidney transplantation has fluctuated over the past 10 years, reaching a peak in 2017, with a generally stable trend.

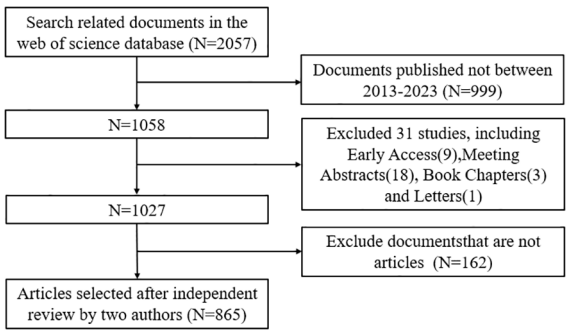


FIGURE 1
Screening flowchart for inclusion of studies.

3.2 Distribution of countries/regions and institution

As shown in Figure 3A, China is the most published country, followed by the United States, Germany, Netherlands and Japan. Afterward, we filtered and visualized all countries based on the number of publications greater than or equal to 2, and built a collaboration network (Figure 3B). We discovered that there are many positive collaborations between different countries. For example, China has close collaborations with the United States; the United States has also actively collaborated with Australia, Japan, France, and the United Kingdom. As shown in the figure, the top six universities come from five countries, with one-third of them located in China. The six universities that have published the most relevant papers are Harvard Medical School, Leiden University, Nanjing Medical University, Oslo University Hospital, Pomeranian Medical University, and Sichuan University. In the last decade, the number of papers published in China has increased rapidly year by year, followed by France (Figure 4).

3.3 Authors and institutions of relevant articles

Among these publications, the Chinese authors published the most papers, followed by the United States (Figure 5A). We constructed a collaborative network based on authors with a number of publications greater than or equal to two. The largest nodes and the most relevant publications, and they were closely related to each other (Figure 5B).

3.4 Analysis of co-cited references and reference burst

When two or more references are cited in more than one article, the two references are considered to be in a co-citation relationship (18). The most cited country was China with 3,877 citations, followed by the United States with 3,306 citations (Figure 6A). The main cited relevant institution was Nanjing Medical University with 47 articles, followed by Pomeranian Medical University with 43 articles (Figure 6B).

A citation burst is a document that is frequently cited by scholars in a particular field over a certain period of time. In our study, CiteSpace identified ten documents with strong citation bursts (Figure 6C). The earliest citation bursts for references appeared in 2015 and the latest in 2021. The literature with the strongest citation outbreak (strength = 5.28) is "The Banff 2017 Kidney Meeting Report: Revised diagnostic criteria for chronic active T cell-mediated rejection, antibody-mediated rejection, and prospects for integrative endpoints for next-generation clinical trials" (19), citing an outbreak period of 2019–2023. Overall, the outbreak strength of the ten publicatio.

3.5 Keywords used in co-citation networks

Different visual clusters of keywords used in published articles were mapped using VOSview and CiteSpace (20). Clustered network visualizations and frequency heat maps of keywords were created on VOSview. CiteSpace was connected to the carrot 2 system to analyze the key topics and related common words, which were shown as follows: kidney transplantation, cytokines, rapid kidney injury, mesenchymal stem cells, and immunosuppression (Figures 7A–C). The yellower color represents the latest hot keywords. We used CiteSpace software to complete the analysis of keyword bursts in the immune microenvironment of kidney transplantation (Figure 7D). "TGF-β", "macrophage" and "antibody-mediated rejection" appeared earlier and were noticed earlier. The keywords with the strongest

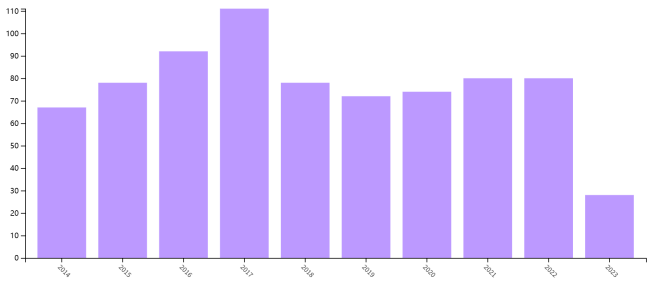


FIGURE 2
Annual yield of research on the immune microenvironment related to survival after kidney transplantation.

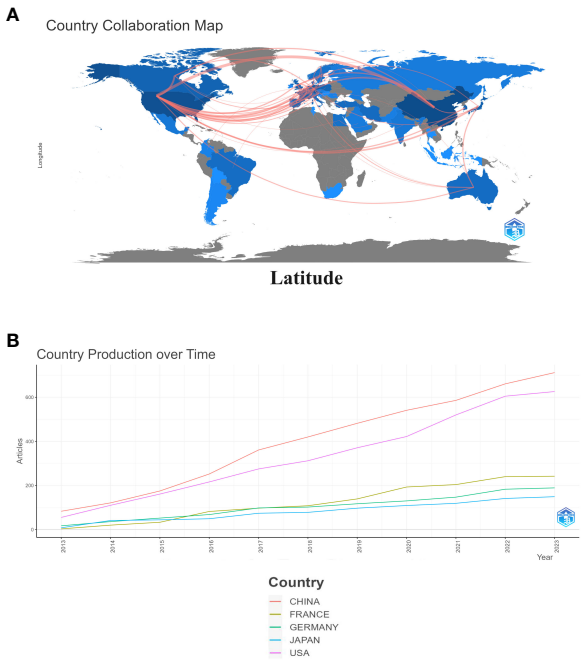


FIGURE 3
Map of collaboration between different countries **(A)**. Trends in publication distribution in the top five countries **(B)**.

cited outbreaks were even transplantation (strength=5.84), TGF-β (strength=5.61) and macrophages (strength=5.14). Macrophage is the keyword with stronger outbreaks that appeared in 2018, which could be a hotspot for research or a turning point with prospective research implication.

4 Discussion

4.1 General information study

This is the first bibliometric and visual analysis of the immune microenvironment in kidney transplantation between 2013 and 2023. A total of 865 articles from SCI-E were included in this study, and each retrieved article was screened to ensure relevance to the topic. The publications and citation frequency related to the immune microenvironment after kidney transplantation have shown a consistent increase, making it an active research topic over the last decade (Figure 2). China, the United States, and Germany were major contributors to this research area. China published the most cited papers, indicating that it has conducted in-depth research in this area (Figure 3). Among the top six selected institutions, the United States institutions mainly collaborated with German research institutions.

China and the United States are the main countries conducting research on the immune microenvironment of kidney transplantation, with China in the first place. About one-third of the top 6 research organizations are located in China, followed by the United States. We have noticed close cooperation between the four countries - the United States, China, Germany and Japan. In terms of authors, there are good collaborations between some authors, such as Ruoyun Tan, Li Sun, Zhen Xu, Min Gu, and Zijie Wang. One of the most influential is the article published in Frontiers in immunology in 2021 and 2022. It is entitled “Combined Immunotherapy With Belatacept and BTLA Overexpression Attenuates Acute Rejection Following Kidney Transplantation” and “Diagnostic Biomarkers and Immune Infiltration in Patients With T Cell-Mediated Rejection After Kidney Transplantation.” They focused on the role played by T-lymphocytes in the immune microenvironment after kidney transplantation in mediating transplant rejection and its clinical use (13, 21).

In terms of institutions, we find that Nanjing Medical University has the most publications. The authors, Ruoyun Tan, Li Sun Min Gu, and Zijie Wang, are from Nanjing Medical University. China and the United States as major countries for research, but the breadth and strength of inter-institutional collaborations are not ideal. Clearly, this situation will hinder the development of the research field in the long run. Therefore, we strongly recommend that research institutions in various countries develop extensive cooperation and communication to promote the development of the immune microenvironment in kidney transplantation.

4.2 Hotspots and Frontiers

The basic structure of research in the field of the immune microenvironment after kidney transplantation can be revealed using literature co-citation networks and keyword clustering

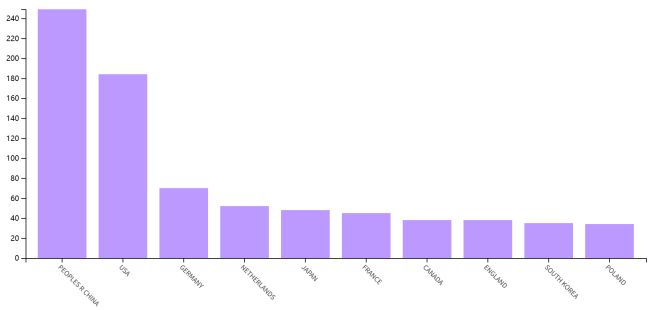


FIGURE 4
Top 10 countries in terms of articles published in the field between 2013-2023.

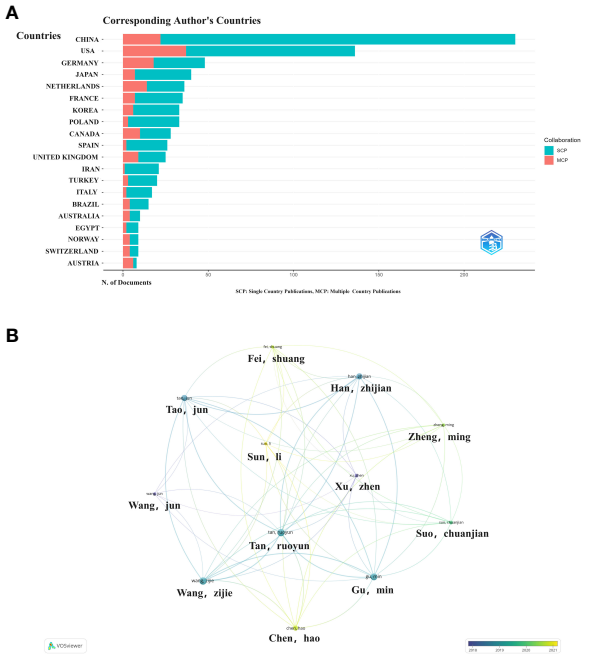


FIGURE 5
Collaborative networks between authors and between countries **(A)**. Countries associated with authorsVisualization map between authors **(B)**.

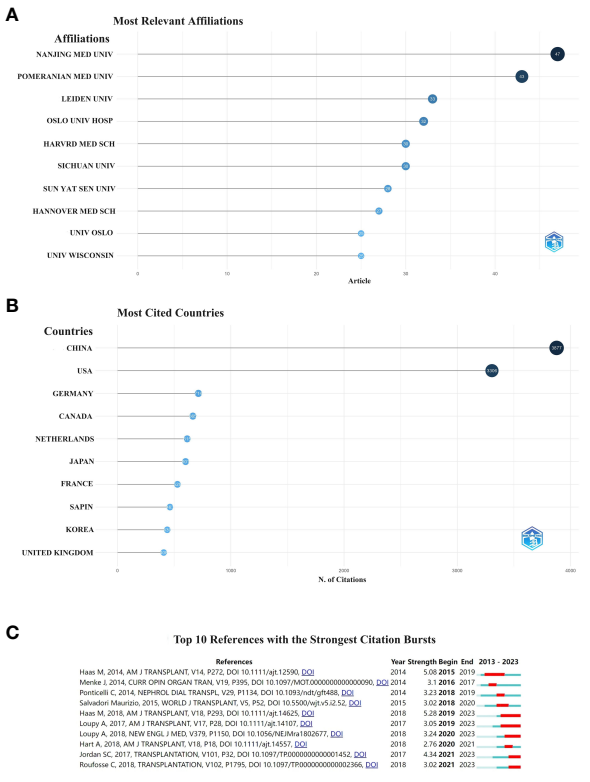


FIGURE 6
Institution **(A)**, country **(B)** and the 10 most cited references to which the publication relates. The red bar indicates the year with the most citations **(C)**.

analyses. The strongest references citation bursts were the meeting summaries of the 12th and 13th Banff Transplant Pathology Conferences. The goal of both meetings hopes to provide a greater understanding of graft immune rejection through the continued integration of advances in histologic, serologic, and molecular diagnostic techniques. To provide precise comprehensive scoring, accurate and routine diagnostics for clinical trials (22, 23). By scrutinizing these analyses, a great deal of valuable information can be gleaned, including TGF-β, and macrophages and antibody-mediated rejection. These findings help to identify emerging trends and research hotspots in the field of the immune microenvironment after kidney transplantation.

4.2.1 Kidney transplantation and macrophages

Macrophages are a key immune system for innate immunity and have a wide range of tissue-resident cell surface receptors, including pattern recognition receptors for damage-associated molecular patterns (DAMPs), complement products, chemokines, Fc fragments, and toll-like receptors (TLRs) (17). It is well known that macrophages play a key role in organogenesis, tissue homeostasis and promotion of tissue injury.

A unique feature of macrophages in allogeneic transplantation is that donor macrophages are transferred with the donor organ at the time of transplantation and recipient monocyte-derived macrophages are subsequently recruited into the allogeneic graft (18). In early severe renal rejection transplants, macrophages account for approximately 60% of the immune cells. Macrophages play a key role in acute cell-mediated rejection and antibody-mediated rejection (24, 25). The major cause of long-term renal transplant failure is histologic interstitial fibrosis and tubular atrophy. The current study found that intercellular communication between renal parenchymal cells and donor-derived macrophages, detected several years after transplantation, plays a key role in the proliferation of damage (19, 20).

In summary, there is growing evidence of the important role of macrophages in tissue inflammation and repair. In recent years, there has been a renewed and increasing emphasis on macrophages. Thus, macrophages are promising therapeutic targets for clinical transplantation (26). Currently, regulatory cell therapy, which aims to protect the immunomodulation of organ grafts, has become an attractive therapeutic approach (27). This approach focuses on expanding specific populations of regulatory immune cells *in vitro* in the form of cell-based medicinal products (CBMPs), which are then infused into transplant recipients to minimize graft rejection. The CBMPs studied so far mainly consist of two polyclonal T regulatory (pTreg-1 and pTreg-2), two donor antigen-reactive Treg (darTreg-CSB and darTreg-sBC), a tolerogenic dendritic cell (autologous tolerogenic dendritic cell [ATDC]) and a regulatory macrophage (Mreg) cell product (28). The current study found that compared to immunosuppressants, regulatory cell therapy has good efficacy in both early and late kidney transplantation with fewer infectious complications and side effects. This is serving as a major direction for future research (27–32).

4.2.2 Kidney transplantation and TGF-β

Cytokines play a key role in coordinating the immune response after kidney transplantation. Therefore, it is crucial to understand the

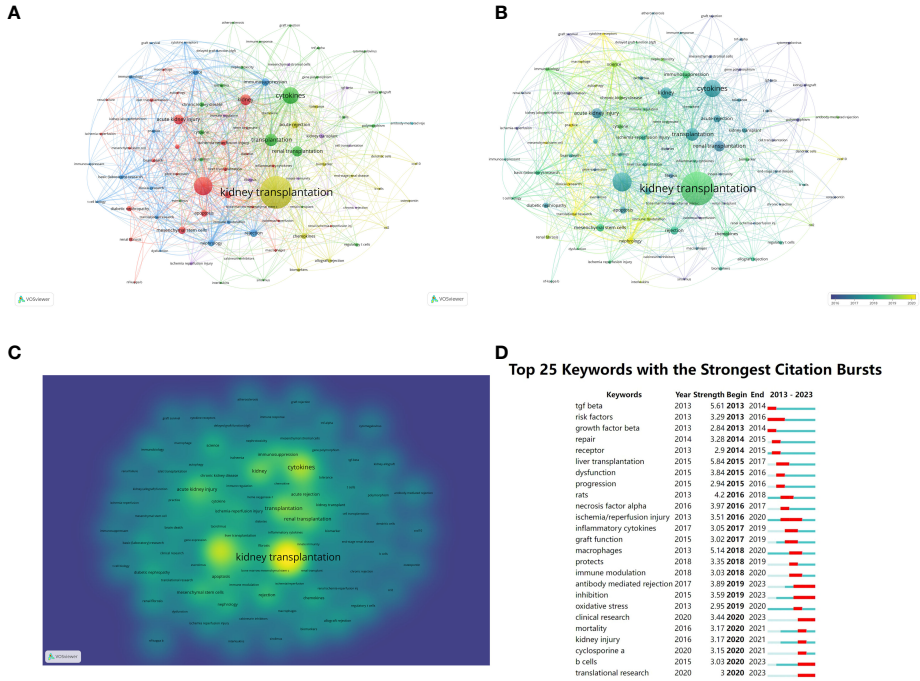


FIGURE 7
Clustering of keywords in studies related to the immune microenvironment and survival after kidney transplantation **(A–C)**. Top 25 most cited keywords **(D)**.

role of cytokines in the allogeneic immune response (33). Among all cytokines, TGF-β is a multifaceted cytokine that regulates pro- and anti-inflammatory responses depending on the microenvironment and target cell type (34). In addition, TGF-β signaling regulates a broad spectrum of biological processes involved in tissue homeostasis and injury responses, including cell growth and differentiation, migration, survival and death (35). To date, three major isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3) encoding for TGFB1, TGFB2 and TGFB3, respectively, have been identified in humans. Of these, TGF-β1 is the most common and best characterized isoform (10).

Interstitial fibrosis is an important factor in graft loss in chronic transplant kidney injury (19). TGF-β1 is a key fibrotic cytokine involved in fibrosis in a variety of chronic kidney and other organ diseases (20). Expression of TGF-β can be detected in allograft patients, especially in failed kidney graft tissues (36).

In kidney transplantation, TGF-β1 has been a topic of interest and most investigators believe that TGF-β1 affects allograft survival in different ways (37). It has been shown that TGF-β1 cells are closely associated with the short-term prognosis of clinical kidney transplantation. Several clinical studies have found that elevated serum TGF-β1 levels after long-term kidney transplantation may have a positive effect on long-term graft survival and may be a predictor of graft survival and function (21, 24–26).

4.3 Kidney transplantation and cell therapy

The combination of general immunosuppressive drugs improves graft survival cycles. However, graft survival has been shortened by chronic rejection and immunosuppressive side effects and has been stagnant for the past decade (1, 38). To address this problem, organ transplantation urgently requires new strategies to reduce our dependence on immunosuppressive drugs to prevent allograft rejection. Currently, the use of cell-based drug products is the state-of-the-art method to reduce immunosuppression in organ transplantation (28). Regulatory cell therapy has emerged as an attractive therapeutic approach to establish immunomodulation aimed at protecting organ grafts (39–41). Currently, common types of regulatory cell therapy include regulatory T cells (Treg), monocyte-derived dendritic cells, and regulatory macrophages. Of these, regulatory T cells are most commonly utilized in clinical practice (42–44). Other cell therapies are currently in clinical testing (45, 46). Current studies have shown that cell therapy is safe and has fewer infectious complications. Thus, immune cell therapy is a potentially useful treatment for renal transplant recipients, reducing the burden of general immunosuppression as well as improving long-term outcomes (28, 47).

4.4 Advantages and shortcomings

This study has several unique advantages over traditional reviews. First, we systematically analyzed the studies on the correlation between immune microenvironment and survival after kidney transplantation for the first time by using bibliometric methods. Second, the bibliometric analysis objectively and comprehensively quantifies and evolves the research hotspots and trends in a certain field through mathematical techniques, which can provide a

comprehensive guide for scholars concerned with related research. Finally, in this review, not only the evidence of hotspots and trends of the correlation between immune microenvironment and survival after kidney transplantation is objectively presented, but also the current research results and outlook are systematically summarized. Therefore, it is hoped that the summarization of the existing research results will help researchers to quickly identify their strengths and weaknesses, thus promoting the development of the field.

Of course, but there are still some limitations that may affect its findings. First, the data used in this paper are exclusively from the WoSCC database, excluding other databases, which may have missed some relevant studies. Second, we only analyzed literature published in English, ignoring studies in other languages. Although the search terms related to the immune microenvironment contained most of the content, they were still lacking, leading to potentially biased results. Some of the relevant literature was not included in the study. The year 2023 is not yet finished ending and only currently published literature was included, which may have excluded some valuable information. Finally, only articles were included without considering political and social publications such as reviews, editorials and books.

5 Conclusion

The immune microenvironment after kidney transplantation has important research value and applications for patient survival. This study utilized the CiteSpace software to evaluate potential collaborators and collaborating institutions, status, and cutting-edge new ideas, thus providing future research trends for exploring and developing the relevance of the immune microenvironment in survival after kidney transplantation. China and the United States have been the leading countries in the last decade. Many studies have shown that immune cells play an important role in the immune microenvironment of kidney transplantation, providing a new therapeutic direction for immunosuppression after kidney transplantation. Overall, the results of this study provide valuable information for guiding future research.

Author contributions

C-LH: Writing – original draft. X-YF: Writing – original draft, Writing – review & editing. YF: Writing – original draft. X-KL: Writing – original draft. YS: Writing – review & editing, Writing – original draft. X-LM: Writing – review & editing. S-WL: Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by Medical Science and Technology Project of Zhejiang Province (2024KY1788), Major Research Program of Taizhou Enze Medical Center Grant (19EZZDA2), Program of Taizhou Science and Technology Grant (23ywa33), Open Project Program of Key Laboratory of Minimally Invasive Techniques and Rapid Rehabilitation of Digestive System Tumor of Zhejiang Province (21SZDSYS01&21SZDSYS09).

Acknowledgments

We thank all the authors who participated in this study.

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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

References

1. Wekerle T, Segev D, Lechler R, Oberbauer R. Strategies for long-term preservation of kidney graft function. *Lancet (London England)*. (2017) 389:2152–62. doi: 10.1016/S0140-6736(17)31283-7

2. Pontrelli P, Grandaliano G, Van Kooten C. Editorial: kidney transplantation and innate immunity. *Front Immunol*. (2020) 11:603982. doi: 10.3389/fimmu.2020.603982

3. Augustine J. Kidney transplant: New opportunities and challenges. *Cleveland Clinic J Med*. (2018) 85:138–44. doi: 10.3949/ccjm.85gr.18001

4. Hariharan S, Israni AK, Danovitch G. Long-term survival after kidney transplantation. *New Engl J Med*. (2021) 385:729–43. doi: 10.1056/NEJMra2014530

5. Ferreira LD, Goff C, Kamepalli S, Montgomery AE, Miggins JJ, Goss JA, et al. Survival benefit of solid-organ transplantation: 10-year update. *Digestive Dis Sci*. (2023) 68:3810–7. doi: 10.1007/s10620-023-08012-1

6. Jordan SC, Ammerman N, Choi J, Huang E, Peng A, Sethi S, et al. The role of novel therapeutic approaches for prevention of allosensitization and antibody-mediated rejection. *Am J Transplant*. (2020) 20 Suppl 4:42–56. doi: 10.1111/ajt.15913

7. Jordan SC, Ammerman N, Choi J, Huang E, Peng A, Sethi S, et al. Novel therapeutic approaches to allosensitization and antibody-mediated rejection. *Transplantation*. (2019) 103:262–72. doi: 10.1097/TP.0000000000002462

8. Becker LE, Weritz B, Yi X, Gross-Weissmann ML, Waldherr R, Zeier M, et al. Evolution of allograft fibrosis and function in kidney transplant recipients: a retrospective analysis of stable patients under CNI and mTORi. *Transplant Int*. (2015) 28:553–64. doi: 10.1111/tri.2015.28.issue-5

9. Quaglia M, Dellepiane S, Guglielmetti G, Merlotti G, Castellano G, Cantaluppi V. Extracellular vesicles as mediators of cellular crosstalk between immune system and kidney graft. *Front Immunol*. (2020) 11:74. doi: 10.3389/fimmu.2020.00074

10. Poppelaars F, Gaya da Costa M, Faria B, Eskandari SK, Damman J, Seelen MA. A functional TGFB1 polymorphism in the donor associates with long-term graft survival after kidney transplantation. *Clin Kidney J*. (2022) 15:278–86. doi: 10.1093/ckj/sfab175

11. Jordan SC, Ammerman N, Choi J, Kumar S, Huang E, Toyoda M, et al. Interleukin-6: an important mediator of allograft injury. *Transplantation*. (2020) 104:2497–506. doi: 10.1097/TP.0000000000003249

12. Cortés-Hernández A, Alvarez-Salazar EK, Arteaga-Cruz S, Rosas-Cortina K, Linares N, Alberú Gómez JM, et al. Highly purified alloantigen-specific tregs from healthy and chronic kidney disease patients can be long-term expanded, maintaining a suppressive phenotype and function in the presence of inflammatory cytokines. *Front Immunol*. (2021) 12:686530. doi: 10.3389/fimmu.2021.686530

13. Arteaga-Cruz S, Cortés-Hernández A, Alvarez-Salazar EK, Rosas-Cortina K, Aguilera-Sandoval C, Morales-Buenrostro LE, et al. Highly purified and functionally stable in *vitro* expanded allospecific Tr1 cells expressing immunosuppressive graft-homing receptors as new candidates for cell therapy in solid organ transplantation. *Front Immunol*. (2023) 14:1062456. doi: 10.3389/fimmu.2023.1062456

14. Guler AT, Waaijer CJ, Palmblad M. Scientific workflows for bibliometrics. *Scientometrics*. (2016) 107:385–98. doi: 10.1007/s11192-016-1885-6

15. Shah SM, Ahmad T, Chen S, Yuting G, Liu X, Yuan Y. A bibliometric analysis of the one hundred most cited studies in psychosomatic research. *Psychother Psychosom*. (2021) 90:425–30. doi: 10.1159/000516185

16. Han X, Zhang J, Chen S, Yu W, Zhou Y, Gu X. Mapping the current trends and hotspots of vascular cognitive impairment from 2000-2021: A bibliometric analysis. *CNS Neurosci Ther*. (2023) 29:771–82. doi: 10.1111/cns.14026

17. Zhang W, Zhang S, Dong C, Guo S, Jia W, Jiang Y, et al. A bibliometric analysis of RNA methylation in diabetes mellitus and its complications from 2002 to 2022. *Front Endocrinol*. (2022) 13:997034. doi: 10.3389/fendo.2022.997034

18. Luo H, Cai Z, Huang Y, Song J, Ma Q, Yang X, et al. Study on pain catastrophizing from 2010 to 2020: A bibliometric analysis *via* citeSpace. *Front Psychol*. (2021) 12:759347. doi: 10.3389/fpsyg.2021.759347

19. Haas M, Loupy A, Lefaucheur C, Roufosse C, Glotz D, Seron D, et al. The Banff 2017 Kidney Meeting Report: Revised diagnostic criteria for chronic active T cell-mediated rejection, antibody-mediated rejection, and prospects for integrative endpoints for next-generation clinical trials. *Am J Transplant*. (2018) 18:293–307. doi: 10.1111/ajt.14625

20. Chen Y, Chen Y, Tan S, Zheng Y, Liu S, Zheng T, et al. Visual analysis of global research on immunotherapy for gastric cancer: A literature mining from 2012 to 2022. *Hum Vaccines immunotherapeutics*. (2023) 19:2186684. doi: 10.1080/21645515.2023.2186684

21. Zhang H, Wang Z, Zhang J, Gui Z, Han Z, Tao J, et al. Combined immunotherapy with belatacept and BTLA overexpression attenuates acute rejection following kidney transplantation. *Front Immunol*. (2021) 12:618737. doi: 10.3389/fimmu.2021.618737

22. Loupy A, Haas M, Solez K, Racusen L, Glotz D, Seron D, et al. The banff 2015 kidney meeting report: current challenges in rejection classification and prospects for adopting molecular pathology. *Am J Transplant*. (2017) 17:28–41. doi: 10.1111/ajt.14107

23. Haas M, Sis B, Racusen LC, Solez K, Glotz D, Colvin RB, et al. Banff 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions. *Am J Transplant*. (2014) 14:272–83. doi: 10.1111/ajt.12590

24. Malone AF. Monocytes and macrophages in kidney transplantation and insights from single cell RNA-seq studies. *Kidney360*. (2021) 2:1654–9. doi: 10.34067/KID.0003842021

25. Mirzakhani M, Shahbazi M, Oliaei F, Mohammadnia-Afrouzi M. Immunological biomarkers of tolerance in human kidney transplantation: An updated literature review. *J Cell Physiol*. (2019) 234:5762–74. doi: 10.1002/jcp.27480

26. Panzer SE. Macrophages in transplantation: A matter of plasticity, polarization, and diversity. *Transplantation*. (2022) 106:257–67. doi: 10.1097/TP.0000000000003804

27. Leclerc S, Lamarche C. Cellular therapies in kidney transplantation. *Curr Opin Nephrol hypertension*. (2021) 30:584–92. doi: 10.1097/MNH.0000000000000737

28. Sawitzki B, Harden PN, Reinke P, Moreau A, Hutchinson JA, Game DS, et al. Regulatory cell therapy in kidney transplantation (The ONE Study): a harmonised design and analysis of seven non-randomised, single-arm, phase 1/2A trials. *Lancet (London England)*. (2020) 395:1627–39. doi: 10.1016/S0140-6736(20)30167-7

29. Schaier M, Morath C, Wang L, Kleist C, Opelz G, Tran TH, et al. Five-year follow-up of a phase I trial of donor-derived modified immune cell infusion in kidney transplantation. *Front Immunol*. (2023) 14:1089664. doi: 10.3389/fimmu.2023.1089664

30. Morath C, Schmitt A, Zeier M, Schmitt M, Sandra-Petrescu F, Opelz G, et al. Cell therapy for immunosuppression after kidney transplantation. *Langenbeck's Arch Surg*. (2015) 400:541–50. doi: 10.1007/s00423-015-1313-z

31. Hendriks SH, Heidt S, Schulz AR, de Fijter JW, Reinders MEJ, Koning F, et al. Peripheral blood immune cell composition after autologous MSC infusion in kidney transplantation recipients. *Transplant Int*. (2023) 36:11329. doi: 10.3389/ti.2023.11329

32. Lai C, Chadban SJ, Loh YW, Kwan TK, Wang C, Singer J, et al. Targeting inflammatory monocytes by immune-modifying nanoparticles prevents acute kidney allograft rejection. *Kidney Int*. (2022) 102:1090–102. doi: 10.1016/j.kint.2022.06.024

33. Orandi BJ, Lonze BE, Jackson A, Terezakis S, Kraus ES, Alachkar N, et al. Splenic irradiation for the treatment of severe antibody-mediated rejection. *Am J Transplant*. (2016) 16:3041–5. doi: 10.1111/ajt.13882

34. Campistol JM, Iñigo P, Larios S, Bescos M, Oppenheimer F. Role of transforming growth factor-beta1 in the progression of chronic allograft nephropathy. *Nephrology dialysis Transplant*. (2001) 16 Suppl 1:114–6. doi: 10.1093/ndt/16.suppl_1.114

35. Kayhan M, Vouillamoz J, Rodriguez DG, Bugarski M, Mitamura Y, Gschwend J, et al. Intrinsic TGF-β signaling attenuates proximal tubule mitochondrial injury and inflammation in chronic kidney disease. *Nat Commun*. (2023) 14:3236. doi: 10.1038/s41467-023-39050-y

36. Willet JD, Pichitsiri W, Jenkinson SE, Brain JG, Wood K, Alhasan AA, et al. Kidney transplantation: analysis of the expression and T cell-mediated activation of latent TGF-β. *J leukocyte Biol*. (2013) 93:471–8. doi: 10.1189/jlb.0712324

37. Du XX, Guo YL, Yang M, Yu Y, Chang S, Liu B, et al. Relationship of transforming growth factor-βl and arginase-1 levels with long-term survival after kidney transplantation. *Curr Med Sci*. (2018) 38:455–60. doi: 10.1007/s11596-018-1900-7

38. Zaza G, Leventhal J, Signorini L, Gambaro G, Cravedi P. Effects of antirejection drugs on innate immune cells after kidney transplantation. *Front Immunol*. (2019) 10:2978. doi: 10.3389/fimmu.2019.02978

39. Safinia N, Grageda N, Scottà C, Thirkell S, Fry LJ, Vaikunthanathan T, et al. Cell therapy in organ transplantation: our experience on the clinical translation of regulatory T cells. *Front Immunol*. (2018) 9:354. doi: 10.3389/fimmu.2018.00354

40. Mansourabadi AH, Mohamed Khosroshahi L, Noorbakhsh F, Amirzargar A. Cell therapy in transplantation: A comprehensive review of the current applications of cell therapy in transplant patients with the focus on Tregs, CAR Tregs, and Mesenchymal stem cells. *Int Immunopharmacol*. (2021) 97:107669. doi: 10.1016/j.intimp.2021.107669

41. Terry LV, Oo YH. The next frontier of regulatory T cells: promising immunotherapy for autoimmune diseases and organ transplantations. *Front Immunol*. (2020) 11:565518. doi: 10.3389/fimmu.2020.565518

42. Zwang NA, Leventhal JR. Cell therapy in kidney transplantation: focus on regulatory T cells. *J Am Soc Nephrol JASN*. (2017) 28:1960–72. doi: 10.1681/ASN.2016111206

43. Mukhin VE, Polyakova YV, Kaabak MM, Babenko NN, Bryzgalina EV, V'Yunkova Y N. Control and prevention of kidney transplant rejection: the role and possibilities for the clinical use of regulatory T-cells in transplantation. *Khirurgiia*. (2019) 9):80–5. doi: 10.17116/hirurgia201909180

44. Bei KF, Moshkelgosha S, Liu BJ, Juvet S. Intragraft regulatory T cells in the modern era: what can high-dimensional methods tell us about pathways to allograft acceptance? *Front Immunol*. (2023) 14:1291649. doi: 10.3389/fimmu.2023.1291649

45. Moreau A, Kervella D, Bouchet-Delbos L, Braudeau C, Saïagh S, Guérif P, et al. A Phase I/IIa study of autologous tolerogenic dendritic cells immunotherapy in kidney transplant recipients. *Kidney Int*. (2023) 103:627–37. doi: 10.1016/j.kint.2022.08.037

46. Nakamura Y, Inoue T. Tolerogenic dendritic cells: promising cell therapy for acute kidney injury. *Kidney Int*. (2023) 104:420–2. doi: 10.1016/j.kint.2023.06.015

47. Harden PN, Game DS, Sawitzki B, van der Net JB, Hester J, Bushell A, et al. Feasibility, long-term safety, and immune monitoring of regulatory T cell therapy in living donor kidney transplant recipients. *Am J Transplant*. (2021) 21:1603–11. doi: 10.1111/ajt.16395

frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 04 April 2024
DOI 10.3389/fimmu.2024.1383936

OPEN ACCESS

EDITED BY
Lisha Mou,
Shenzhen Second People's Hospital, China

REVIEWED BY
Jeanette Villanueva,
Victor Chang Cardiac Research Institute, Australia
Xiaofeng Zhang,
Greenwich Hospital/Yale New Haven Health, United States

*CORRESPONDENCE
Yi Wang
w_yi2022@163.com
Zhengteng Yang
330560167@qq.com

†These authors have contributed equally to this work

RECEIVED 08 February 2024
ACCEPTED 15 March 2024
PUBLISHED 04 April 2024

CITATION
Sun Q, Song S-Y, Ma J, Li D, Wang Y, Yang Z and Wang Y (2024) Cutting edge of genetically modified pigs targeting complement activation for xenotransplantation.
Front. Immunol. 15:1383936.
doi: 10.3389/fimmu.2024.1383936

Cutting edge of genetically modified pigs targeting complement activation for xenotransplantation

Qin Sun[1†], Si-Yuan Song[2†], Jiabao Ma[3†], Danni Li[4†], Yiping Wang[5], Zhengteng Yang[3*] and Yi Wang[6*]

[1]Department of Endocrinology, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, School of Medicine, University of Electronic Science and Technology of China, Chengdu, China, [2]Department of Neuroscience, Baylor College of Medicine, Houston, TX, United States, [3]School of Pharmacy, Guangxi University of Chinese Medicine, Nanning, China, [4]Department of Pharmacy, Longquanyi District of Chengdu Maternity & Child Health Care Hospital, Chengdu, China, [5]Department of Critical Care Medicine, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, School of Medicine, University of Electronic Science and Technology of China, Chengdu, China, [6]Clinical Immunology Translational Medicine Key Laboratory of Sichuan Province, Center of Organ Transplantation, Sichuan Academy of Medical Science and Sichuan Provincial People's Hospital, Chengdu, Sichuan, China

In the quest to address the critical shortage of donor organs for transplantation, xenotransplantation stands out as a promising solution, offering a more abundant supply of donor organs. Yet, its widespread clinical adoption remains hindered by significant challenges, chief among them being immunological rejection. Central to this issue is the role of the complement system, an essential component of innate immunity that frequently triggers acute and chronic rejection through hyperacute immune responses. Such responses can rapidly lead to transplant embolism, compromising the function of the transplanted organ and ultimately causing graft failure. This review delves into three key areas of xenotransplantation research. It begins by examining the mechanisms through which xenotransplantation activates both the classical and alternative complement pathways. It then assesses the current landscape of xenotransplantation from donor pigs, with a particular emphasis on the innovative strides made in genetically engineering pigs to evade complement system activation. These modifications are critical in mitigating the discordance between pig endogenous retroviruses and human immune molecules. Additionally, the review discusses pharmacological interventions designed to support transplantation. By exploring the intricate relationship between the complement system and xenotransplantation, this retrospective analysis not only underscores the scientific and clinical importance of this field but also sheds light on the potential pathways to overcoming one of the major barriers to the success of xenografts. As such, the insights offered here hold significant promise for advancing xenotransplantation from a research concept to a viable clinical reality.

KEYWORDS

xenotransplantation, complement systems, genetically modified pigs, C3a, C3b

1 Introduction

As of 2019, China's organ donor registration boasted close to 1.7 million volunteers, a testament to its advancements in the field of organ transplantation. In that same year, China ranked as the world's second-largest provider of allogeneic transplants, showcasing over 10,000 kidney and 5,000 liver transplants at the 4th China-International Organ Donation Conference (1). A significant policy shift in 2015 marked the transition to voluntary organ donations from Chinese citizens as the exclusive legal source for transplants (2), which, despite its ethical merits, has led to an even greater deficit in available human organs for transplantation and hindered research due to the scarcity.

This backdrop has propelled xenotransplantation to the forefront as a promising solution to this shortage. Research in this domain has progressively moved toward identifying specific donor species, with primates being an initial choice due to their genetic closeness to humans. However, the use of baboon organs has consistently resulted in patient fatalities (3), steering the scientific focus toward pigs as suitable organ donors. Pigs, with their comparable organ size to humans and favorable breeding traits, are currently the focal point of xenotransplantation research (4–6). The journey of xenotransplantation, illustrated in Figure 1, is now directed toward the development of transgenic pigs, which are being heralded as the next step in transplantation science.

The hyperacute rejection of transplants, primarily driven by the complement system, has been a longstanding challenge. This system's activation leads to the production of active compounds like C3a and C3b (7), which catalyze immune inflammation and graft endothelial thromboembolism. The discovery of α-Gal on graft surfaces as a trigger for complement activation has steered the development of α-Gal knockout (α-GalKO) pigs. Chinese research teams, such as the one led by Pandengke, have been at the helm of creating and refining α-Gal and β-Gal knockout pigs for several generations. A milestone was achieved in June 2020 with the cloning of a pig possessing triple knockouts, a significant leap made possible by gene editing technologies targeting the B4GalNT2 and CMAH genes (8).

The crux of this article revolves around the utilization of gene editing to modify pig donors, aiming to mitigate the issue of complement activation-induced hyperacute rejection post-xenotransplantation. We discuss dual approaches to this end: the genetic elimination of the α-Gal epitope from xenograft endothelium and the introduction of human complement regulatory proteins (hCRPs) into grafts via transgenesis. Additionally, we explore the pharmaceutical avenues developed to inhibit the complement system, a critical strategy to counter rejection in xenotransplantation.

2 Xenograft activates the complement system

Xenograft transplantation challenges the human immune system, particularly through the activation of the complement system, a sophisticated network of over 50 proteins crucial for the immune response (9). It can be activated via three primary pathways: the classical pathway (CL), the alternative pathway (AP), and the lectin pathway (MBL) (10), all leading to the potential destruction of the xenograft.

The classical pathway is initiated by the C1 complex binding to antigen-antibody complexes, leading to the activation of C4 and C2, and subsequently, the formation of C3 convertase (11). This enzyme is pivotal in cleaving C3 into C3a and C3b, with C3b joining with C4b2a to form C5 convertase, advancing the complement cascade (12). In contrast, the alternative pathway, triggered by substances like natural polysaccharides, relies on the spontaneous hydrolysis of C3 and the formation of a fluid-phase C3

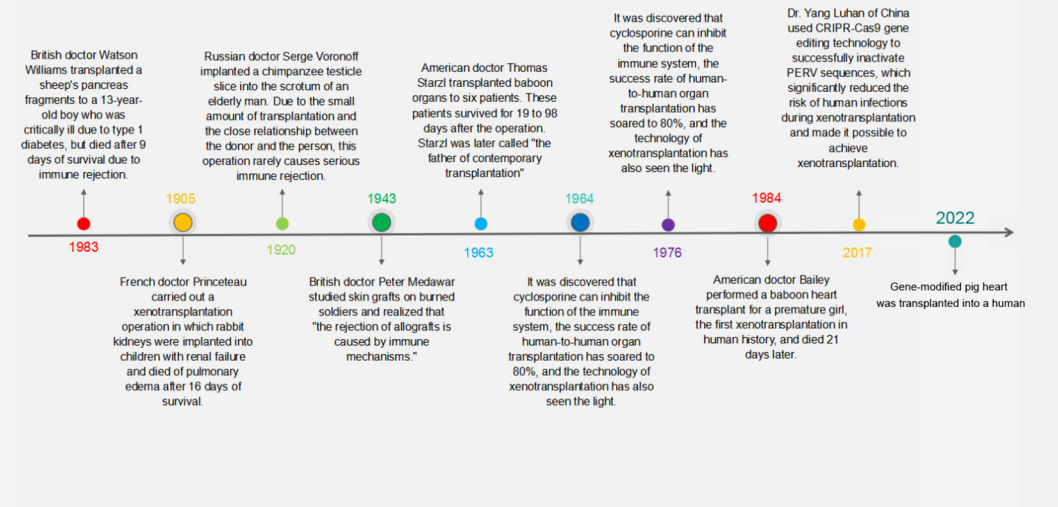


FIGURE 1
Milestones in the history of xenotransplantation. This timeline graphically represents the pivotal moments and groundbreaking achievements in the field of xenotransplantation from the early 20th century to modern day.

convertase, leading to a modest production of C3b that enhances phagocytosis and anaphylatoxin production (13, 14). The lectin pathway starts with MBL binding to microorganism surface carbohydrates, recruiting MASP-1 and MASP-2 to form C3 convertase, mirroring the classical pathway's initial steps (15, 16).

Xenotransplantation, especially from pig donors to primate recipients, introduces immunological hurdles due to the rapid complement-mediated response that often leads to hyperacute rejection (HAR), characterized by graft embolism and failure (7, 17). The presence of natural antibodies in the recipient binding to pig endothelial cell surface glycoproteins, such as α-galactosidase (α-Gal) and N-acetylneuraminic acid hydroxylase (Neu5Gc protein), activates the complement system, leading to clotting, vascular embolism, and graft failure (18, 19). Studies have shown that pig hearts transplanted into baboons are susceptible to this rapid rejection, with serum analysis revealing IgM-α-Gal antibodies bound to α-Gal, triggering the complement activation pathways (18, 19).

However, genetic engineering offers promising strategies to circumvent HAR by modifying donor pigs to reduce the human complement system's activation effects on graft survival. Knocking out genes encoding heterologous endothelial antigens and creating transgenic pigs expressing hCRPs are at the forefront of these strategies (20). *In vitro* studies using pancreatic islets from α-GalKO pigs showed reduced antibody deposition and lower levels of complement activation, suggesting a diminished role of the lectin pathway in xenograft rejection (18, 19).

Further research into the immunological interactions between pig tissues and primate hosts has revealed that even in the absence of preformed natural antibodies, HAR can occur, potentially through the alternative complement pathway (21, 22). This indicates a complex interplay between the classical and alternative pathways in graft rejection, where the alternative pathway may exacerbate C3a deposition within grafts, amplifying inflammatory and immune responses (23).

Complement proteins C3a and C5a, along with the membrane attack complex formed via the classical and alternative pathways, play critical roles in xenograft tissue lysis. These proteins not only mediate inflammation but also activate coagulation cascades, contributing to the risk of thromboembolism in xenografts (24). Studies have shown that inflammation induced by complement activation can significantly reduce the expression of porcine thrombomodulin, an anti-inflammatory molecule, on vascular endothelial cells, highlighting the interconnectedness of inflammation and thrombosis in xenotransplantation (25).

Addressing the challenge of HAR in xenotransplantation requires innovative approaches to prevent complement activation. Genetic modifications in pig donors, such as eliminating α-Gal epitopes and introducing hCRPs, represent vital steps toward improving graft survival and reducing complement-mediated rejection risks. These strategies not only aim to mitigate the immediate immunological challenges but also open new avenues for long-term success in xenotransplantation, potentially transforming it into a viable solution for organ shortages (20).

3 Genetic modifications in pigs

Pigs are optimal donors for xenotransplantation due to their genetic, physiological, and anatomical similarities to humans, alongside their capability for breeding in controlled environments (26, 27). Despite these advantages, the genetic differences between pigs and humans can lead to immunological discordance and potential organ rejection. Advancements in genetic engineering and somatic cell nuclear transfer have facilitated modifications to the pig genome to reduce organ immunogenicity, aiming to prevent the human immune system from rejecting pig organ transplants (27, 28) (Figure 2). This progress is pivotal in addressing immune rejections, with research exploring the growth of human organs within pigs through chimeric methods, although still predominantly in rodent models.

The risk of viral infection, particularly from porcine endogenous retroviruses (PERVs), represents a significant challenge in xenotransplantation (29). Strategies to mitigate this

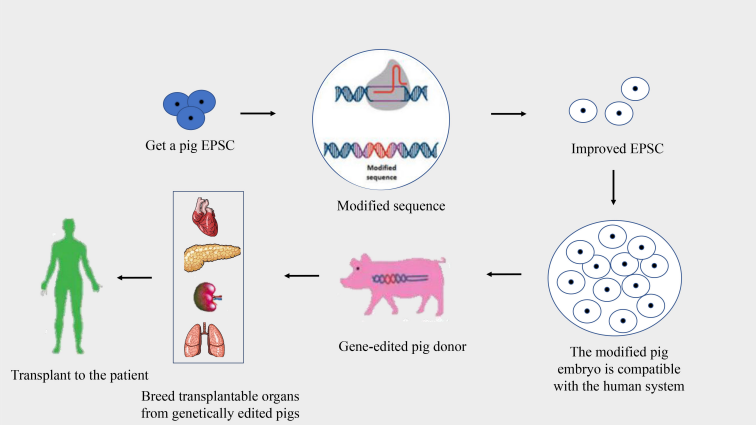


FIGURE 2
Process of creating gene-edited pig donors for xenotransplantation. This flowchart illustrates the stages of developing gene-edited pigs for organ donation to human recipients.

risk include breeding pigs in specific-pathogen-free (SPF) environments and selecting pigs free from PERV-C to reduce the risk of PERV-A/C-mediated transmission to humans (30). Although endogenous retroviruses remain inactive within their host species, causing no apparent disease, they could potentially become active and infectious upon transmission to a recipient (27, 31, 32). Immune molecular incompatibility poses another obstacle, with the immune system targeting foreign grafts, notably triggered by pre-existing natural xenoantibodies recognizing Gal epitopes (33–35). Genetically engineered pigs lacking alpha-1,3-Gal epitopes represent a crucial step toward overcoming HAR and other forms of immune rejection (27, 28).

Non-specific immune reactions, such as the instant blood-mediated inflammatory reaction (IBMIR), significantly challenge xenogeneic islet transplantation, leading to substantial graft loss (36). Addressing these reactions involves genetic modifications of donor animals, anticoagulation therapies, and the use of anti-inflammatory treatments to preserve graft integrity and prevent adaptive immune activation (37).

4 Genetic modification of pigs for xenotransplantation

The development of genetically engineered pigs marks a significant leap forward in addressing the challenges of xenotransplantation from pigs to primates. Through cutting-edge genome editing techniques, scientists have been able to introduce precise modifications into the pig genome to mitigate xenograft rejection and diminish the risk of interspecies infection (28). Among the most promising modifications are the disruption of the α-Gal and the incorporation of hCRPs, which have shown considerable promise in preclinical studies involving pig-to-non-human primate transplants.

Recent breakthroughs in gene editing, powered by artificial nuclease technologies, have significantly expanded the possibilities for generating gene-edited pigs. These technologies, including zinc finger nuclease (ZFN) (38), transcription activator-like effector nuclease (TALEN) (39), and the CRISPR/Cas system (40–43), have enabled not only simple gene knockouts and knock-ins but also complex multi-gene editing, precision point mutations, and conditional gene modifications. These advancements allow for gene editing at various developmental stages of pigs, offering new avenues for creating donor pigs with optimized genetic traits for xenotransplantation.

The hCRPs play a crucial role in maintaining the delicate balance between complement activation and inhibition. Proteins such as decay-accelerating factor (hDAF), membrane cofactor protein (hMCP), and reactive membrane cleavage inhibitor (hCD59) prevent unregulated complement activity, which could otherwise lead to continuous production of complement components and exacerbate endothelial damage in xenografts (44). The expression of these hCRPs in donor pigs can significantly reduce the risk of hyperacute rejection by limiting the formation of the membrane attack complex (MAC) and mitigating complement-mediated damage.

The application of DAF (CD55), a membrane component found on various human cells, has been explored for its potential to protect grafts from early rejection phases (45, 46). DAF can disrupt C3 convertases on the cell surface, effectively downregulating complement activation. Studies have demonstrated that expressing hDAF in pig islets and other tissues can enhance protection against human complement-mediated lysis and extend graft survival (47, 48). Similarly, the expression of human h-transferase, an inhibitor of the alternative complement pathway, has been shown to provide significant protection for xenografts against human complement attack, as evidenced by experiments with transgenic pig livers transplanted into baboons (49, 50). These genetic modifications underscore the potential of genetically engineered pigs to overcome some of the most significant barriers to successful xenotransplantation.

Membrane cofactor protein (MCP, CD46) plays a crucial role in preventing the amplification loop of C3b deposition mediated by alternative convertase. In an innovative approach, researchers employed α-GalKO pigs that were genetically modified to express human CD46 across all tissues, including the heart, exhibiting elevated levels of human CD46 expression. This genetic modification not only prevented B cell infiltration but also significantly reduced T cell activity in the peripheral blood of transplants, indicating an effective suppression of the T cell-mediated response to xenoantigens (51).

Human CD59 serves as a protective mechanism against autologous cell damage by the human complement system, specifically by inhibiting the formation of the membrane attack complex (MAC) during the final stage of complement activation (7, 52). Utilizing embryonic germ (EG) cells, which unlike somatic cells can proliferate indefinitely while remaining undifferentiated, Hosup Shim (53) developed a method to create transgenic pigs capable of expressing human CD59. These EG cells, derived from primordial germ cells (PGC) (54), were genetically modified with a 456 bp fragment of the hCD59 gene, encompassing the entire coding region, obtained from human fibroblast genes (55). Post-transfection into porcine EG cells (56), these modified cells exhibited significantly higher mitochondrial activity when exposed to human serum containing complement, compared to non-transgenic controls, demonstrating enhanced survival under HAR conditions.

The development of multi-transgenic pigs offers a promising strategy to mitigate xenograft damage more effectively. For instance, pig cells expressing human CD59 have shown increased resistance to lysis by human macrophages (57). Furthermore, the expression of α1,2-fucosyltransferase (H-transferase, HT), alongside the knockout of the α1,3-galactosyltransferase (GT) gene, presents a viable alternative strategy. Combining gene edits to express both hCD59 and human HT, or to achieve α-GalKO, enhances the protective effects against human serum, thereby improving cell and organ survival post-transplantation (58). Transgenic pigs expressing human CD55, CD59, and H-Transferase have shown significant reduction in complement-mediated graft destruction (50), although these modifications alone could not completely prevent humoral rejection, characterized by antibody deposition and thrombotic microangiopathy. This suggests that while

significant strides have been made, further research is necessary to minimize rejection mechanisms in xenotransplantation (28).

5 Complement system target drugs for transplantation therapy

The complement system plays a crucial role in innate immunity and immune regulation, protecting against infections and participating in various physiological and pathological processes (59). Despite its protective functions, dysregulated complement activation can contribute to detrimental effects, including inflammation and tissue damage. A deeper understanding of the complement system's components and mechanisms has spurred the development of therapeutic drugs aimed at modulating complement activity. These drugs target various complement pathways, offering potential treatments for infectious, inflammatory, traumatic, cancerous, autoimmune, or age-related conditions, as well as preventing transplant rejection (60).

Eculizumab, the first drug targeting the complement system, has revolutionized the treatment landscape for diseases like paroxysmal nocturnal hemoglobinuria (PNH), significantly improving patient outcomes (59, 61). In the context of organ transplantation, the complement system is implicated in several complications, including ischemia-reperfusion injury and antibody-mediated rejection. Therapeutics such as C1-1NH (Cinryze, Berinert, Ruconest, Cetor) and Soliris are making their way into clinical practice, showing promise but with varying efficacy levels (62). Future research is needed to identify the most effective complement inhibitors and devise optimal treatment strategies. The development programs for inhibitors targeting over a dozen distinct complement pathways are summarized, with some already undergoing clinical trials in both healthy volunteers and patients (62–64). This broad spectrum of complement-targeted therapies underscores the system's significance across a range of medical conditions and its potential as a therapeutic target in transplant medicine, where controlling complement activation could mitigate transplant rejection and improve graft survival.

6 Conclusions and perspective

The critical shortage of human organs for transplantation is a global challenge, and xenotransplantation has emerged as a promising approach to address this dilemma. Genetically engineered pigs are at the forefront of donor options in xenotransplantation, offering a viable solution to the organ shortage crisis. Advances in gene editing technologies, such as CRISPR/Cas9, TALEN, and somatic cell nuclear transfer (SCNT), have significantly propelled xenotransplantation research forward, enabling precise genetic modifications in pig donors.

The complement system plays a dual role in xenotransplantation: it is a key player in the immune response against porcine endothelial cells following the binding of anti-porcine antibodies and contributes to ischemia-reperfusion injury (IRI). Additionally, its involvement in coagulation, inflammation, and the adaptive immune response adds layers of complexity to its function in xenograft rejection. Despite these immunobiological challenges, the advent of genetically modified pigs, alongside an expanding array of immunosuppressants and anti-inflammatory medications, is progressively overcoming the hurdles faced by xenotransplantation.

Current genetic engineering efforts targeting complement regulatory mechanisms have effectively mitigated concerns related to complement activation. However, there remains a potential necessity for anti-complement and anti-inflammatory interventions, especially in acute settings, to ensure the long-term success and acceptance of xenotransplantation as a feasible solution to the organ shortage crisis.

Author contributions

QS: Writing - original draft. QY: Writing – original draft. S-YS: Writing – original draft. JM: Writing – original draft. DL: Writing – original draft. YPW: Writing – original draft. ZY: Funding acquisition, Writing – review & editing. YW: Funding acquisition, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This research was supported by the National Natural Science Foundation of China (81802504), the Sichuan Science and Technology Bureau (2021YFH0380 and 2023YFH0010), and a grant from Guangxi Science and Technology Base and Talent Project (AD23023011).

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.

Publisher's note

References

1. National Bureau of Statistics of China. *National Economy was Generally Stable in 2019 with Main Projected Targets for Development Achieved*, (Beijing, China: National Bureau of Statistics of China) (2020). Available at: https://www.stats.gov.cn/english/PressRelease/202001/t20200117_1723398.html.

2. Li J-H, Xu X, Wang Y-F, Xie H-Y, Chen J-Y, Dong N-G, et al. Chinese expert consensus on organ protection of transplantation (2022 edition). *Hepatobiliary & Pancreatic Diseases International*. (2022) 21(6):516–526. doi: 10.1016/j.hbpd.2022.10.010

3. Cooper DKC. A brief history of cross-species organ transplantation. *Proc (Bayl Univ Med Cent)*. (2012) 25(1):49–57. doi: 10.1080/08998280.2012.11928783

4. Reardon S. First pig-to-human heart transplant: what can scientists learn? *Nature*. (2022) 601:305–6. doi: 10.1038/d41586-022-00111-9

5. Montgomery RA, Stern JM, Lonze BE, Tatapudi VS, Mangiola M, Wu M, et al. Results of two cases of pig-to-human kidney xenotransplantation. *N Engl J Med*. (2022) 386:1889–98. doi: 10.1056/NEJMoa2120238

6. Porrett PM, Orandi BJ, Kumar V, Houp J, Anderson D, Cozette Killian A, et al. First clinical-grade porcine kidney xenotransplant using a human decedent model. *Am J Transplant*. (2022) 22:1037–53. doi: 10.1111/ajt.16930

7. Tan LA, Yu B, Sim FC, Kishore U, Sim RB. Complement activation by phospholipids: the interplay of factor H and C1q. *Protein Cell*. (2010) 001:1033–49. doi: 10.1007/s13238-010-0125-8

8. Zhang Y, Pan D, Sun X, Sun G, Wang X, Liu X, et al. Production of porcine cloned transgenic embryos expressing green fluorescent protein by somatic cell nuclear transfer. *Sci China Ser C*. (2006) 49):1–8. doi: 10.1007/s11427-005-0071-5

9. Liu C-CM, Susan M, Kao AH, Navratil JS, Ahearn JM. Cell-bound complement biomarkers for systemic lupus erythematosus: from benchtop to bedside. *Rheum Dis Clin North Am*. (2010) 36:161–72. doi: 10.1016/j.rdc.2009.12.003

10. Roos A, Daha M. Antibody-mediated activation of the classical complement pathway in xenograft rejection. *Transplant Immunol*. (2002) 9:257–70. doi: 10.1016/S0966-3274(02)00042-4

11. Wallis R, Mitchell DA, Schmid R, Schwaeble WJ, Keeble AH. Paths reunited: initiation of the classical and lectin pathways of complement activation. *Immunobiology*. (2010) 1:1–11. doi: 10.1016/j.imbio.2009.08.006

12. Ballow M. C1-Bypass complement-activation pathway in patiente with chronic urticaria and angiosoelig. *Lancet*. (1975) 306:248–50. doi: 10.1016/S0140-6736(75)90963-0

13. Gtze O, Müller-Eberhard HJ. The alternative pathway of complement activation. *Adv Immunol*. (1976) 24:1–35. doi: 10.1016/s0065-2776(08)60328-4

14. Merle NS, Elizabeth CS, Veronique FB, Roumenina LT. Complement system part I a. *Front Immunol*. (2015) 6:1–30. doi: 10.3389/fimmu.2015.00257

15. Cooper DKC, Sachs DH, Colvin RB, Shimizu A, Hisashi Y, Yamada K, et al. Rejection of cardiac xenografts transplanted from αr,3-3omsplantedFDSLHSCRHJ genesplantedF (GalT-alT pigs to baboons. *Am J Transplant*. (2008) 8:2516–26. doi: 10.1111/j.1600-6143.2008.02444.x

16. Kaplon RJ, Platt JL, Kwiatkowski PA, Edwards NM, Xu HE, Shah AS, et al. Absence of hyperacute rejection in pig-to-primate orthotopic pulmonary xenografts. *Transplantation*. (1995) 59:410. doi: 10.1097/00007890-199502150-00017

17. Chen RH, Kadner A, Mitchell RN, Adams DH. Mechanism of delayed rejection in transgenic pig-to-primate cardiac xenotransplantation. *J Surg Res*. (2000) 90:119–25. doi: 10.1006/jsre.2000.5864

18. Thompson P, Badell I, Lowe M, Cano J, Song M, Leopardi F, et al. Islet xenotransplantation using gal-deficient neonatal donors improves engraftment and function. *other*. (2011) 11:1–20. doi: 10.1111/j.1600-6143.2011.03720.x

19. Broom C, Uknis ME. *Methods of treating antibody-mediated rejection in organ transplant patients with C1-esterase inhibitor*. (Australia: Patent) (2018).

20. Butler JR, Ladowski JM, Martens GR, Tector M, Tector AJ. Recent advances in genome editing and creation of genetically modified pigs. *Int J Surgery*. (2015) 23:217–22. doi: 10.1016/j.ijsu.2015.07.684

21. Suckfüll M, Müdsam M, Pieske O, Enders G, Babic R, Hammer C. Immunohistological studies of complement activation after xenogeneic perfusion of a working heart model. *Transplant Int*. (1994) 7:324–8. doi: 10.1111/j.1432-2277.1994.tb01241.x

22. Forty J, Hasan R, Cary N, White DJ, Wallwork J. Hyperacute rejection of rabbit hearts by human blood is mediated by the alternative pathway of complement. *Transplant Proc*. (1992) 24:488.

23. Platts-Mills TAE, Ishizaka K. Activation of the alternative pathway of human complement by rabbit cells. *J Immunol*. (1974) 113:348–58. doi: 10.4049/jimmunol.113.1.348

24. Li Y, Gong P, Kong C, Tian X. Bufalin engages in RIP1-dependent and ROS-dependent programmed necroptosis in breast cancer cells by targeting the RIP1/RIP3/PGAM5 pathway. *Anti Cancer Drugs*. (2019) 30:e0770. doi: 10.1097/CAD.0000000000000770

25. Ochando J, Ordikhani F, Boros P, Jordan S. The innate immune response to allotransplants: mechanisms and therapeutic potentials. *Cell Mol Immunol*. (2019) 16):350–6. doi: 10.1038/s41423-019-0216-2

26. Heng Z, Kaixiang X, Ninglin F, Hongye Z, Hongjiang W. Construction and current status of gene-edited xenotransplantation pigs. *Electronic J Pract Organ Transplantation*. (2018) 6:412–8.

27. Hryhorowicz M, Zeyland J, Słomski R, Lipiński D. Genetically modified pigs as organ donors for xenotransplantation. *Mol Biotechnol*. (2017) 59(9-10):435–44. doi: 10.1007/s12033-017-0024-9

28. Klymiuk N, Aigner B, Brem G, Wolf E. Genetic modification of pigs as organ donors for xenotransplantation. *Mol Reprod Dev*. (2010) 77:209–21. doi: 10.1002/mrd.21127

29. Specke V, Rubant S, Denner J. Productive infection of human primary cells and cell lines with porcine endogenous retroviruses. *Virology*. (2001) 285:177–80. doi: 10.1006/viro.2001.0934

30. Patience C, Switzer WM, Takeuchi Y, Griffiths DJ, Weiss RA. Multiple groups of novel retroviral genomes in pigs and related species. *J Virol*. (2001) 75:2771–5. doi: 10.1128/JVI.75.6.2771-2775.2001

31. Denner J. Porcine Endogenous Retroviruses and Xenotransplantation. *Viruses*. (2021) 13(11):2156. doi: 10.3390/v13112156

32. Denner J. Recombinant porcine endogenous retroviruses (PERVviruse a new risk for xenotransplantation? *Xenotransplantation*. (2010) 17:120–0. doi: 10.1111/j.1399-3089.2010.00573_21.x

33. Ibrahim Z, Busch J, Awwad M, Wagner R, Wells K, Cooper DKC. Selected physiologic compatibilities and incompatibilities between human and porcine organ systems. *Xenotransplantation*. (2010) 13:488–99. doi: 10.1111/j.1399-3089.2006.00346.x

34. Guoling L, Zhiqian X, Huaqiang Y, Zhenfang W. Research progress of transgenic and gene-edited pigs. *J South China Agric Univ*. (2019) 40(5):91–101.

35. Tanihara F, Hirata M, Otoi T. Current status of the application of gene editing in pigs. *J Reprod Dev*. (2021) 67(3):177–87. doi: 10.1262/jrd.2021-025

36. Matsumoto S, Tomiya M, Sawamoto O. Current status and future of clinical islet xenotransplantation. *J Diabetes*. (2016) 8(4):483–93. doi: 10.1111/1753-0407.12395

37. Zhengzhao L, Tian H, Zhiming C, Lisha M. Research progress of porcine islet xenotransplantation. *Organ transplant*. (2017) 008:246–50.

38. Miller JC, Holmes MC, Wang J, Guschin DY, Lee YL, Rupniewski I, et al. An improved zinc-finger nuclease architecture for highly specific genome editing. *Nat Biotechnol*. (2007) 25:778–85. doi: 10.1038/nbt1319

39. Schmid-Burgk JL, Schmidt T, Kaiser V, Höning K, Hornung V. A ligation-independent cloning technique for high-throughput assembly of transcription activator-like effector genes. *Nat Biotechnol*. (2013) 31(1):76–81. doi: 10.1038/nbt.2460

40. Mali P, Yang L, Esvelt KM, Aach J, Guell M, Dicarlo JE, et al. RNA-guided human genome engineering *via* cas9. *Science*. (2013) 339:823. doi: 10.1126/science.1232033

41. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-cas9 for genome engineering. (2014) 157(6):1262–78. doi: 10.1016/j.cell.2014.05.010

42. Gaj T, Gersbach CA, Barbas CF. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol*. (2013) 31(7):397–405. doi: 10.1016/j.tibtech.2013.04.004

43. Yaoqiang H, Guoling L, Huaqiang Y, Zhenfang W. Application of gene-edited pigs in biomedical research. *Genetic*. (2018) v.40:30–44.

44. Roumenina LT, Zuber J, Frémeaux-Bacchi V. Physiological and therapeutic complement regulators in kidney transplantation. *Curr Opin Organ Transplant*. (2013) 18:421–9. doi: 10.1097/MOT.0b013e32836370ce

45. Kinoshita T. Distribution of decay-accelerating factor in the peripheral blood of normal individuals and patients with paroxysmal nocturnal hemoglobinuria. *J Exp Med*. (1985) 162:75–92. doi: 10.1084/jem.162.1.75

46. Asch AS. Decay-accelerating factor is present on cultured human umbilical vein endothelial cells. *J Exp Med*. (1986) 163:221–6. doi: 10.1084/jem.163.1.221

47. Yamamoto T, Iwase H, King TW, Hara H, Cooper DKC. Skin xenotransplantation: Historical review and clinical potential. *Burns*. (2018) 44 (7):1738–1749. doi: 10.1016/j.burns.2018.02.029

48. Schmidt P, Goto M, Mauff BL, Anegon I, Korsgren O. Adenovirus-mediated expression of human CD55 or CD59 protects adult porcine islets from complement-mediated cell lysis by human serum. *Transplantation*. (2003) 75:697–702. doi: 10.1097/01.TP.0000053249.39753.D6

49. Young-Hee J, Chi-Hun P, Gun-Hyuk J, Yeun-Ik J, In-Sung H, Yeon-Woo J, et al. Production of multiple transgenic yucatan miniature pigs expressing human complement regulatory factors, human CD55, CD59, and H-transferase genes. *PloS One*. (2013) 8:e63241. doi: 10.1371/journal.pone.0063241

50. Ramírez P, Montoya MJ, Ríos A, Palenciano CG, Majado M, Chávez R, et al. Prevention of hyperacute rejection in a model of orthotopic liver xenotransplantation from pig to baboon using polytransgenic pig livers (CD55, CD59, and H-transferase). *Transplant Proc*. (2005) 37:4103–6. doi: 10.1016/j.transproceed.2005.09.186

51. Mohiuddin MM, Corcoran PC, Singh AK, Azimzadeh A, Hoyt RF Jr., Thomas ML, et al. B-cell depletion extends the survival of GTKO.hCD46Tgpig heart xenografts

in baboons for up to 8 months. *Am J Transplantation*. (2012) 12:763–71. doi: 10.1111/j.1600-6143.2011.03846.x

52. Kimberley FC, Sivasankar B, Morgan BP. Alternative roles for CD59. *Mol Immunol*. (2007) 44:73–81. doi: 10.1016/j.molimm.2006.06.019

53. Ahn KS, Ji YW, Park JK, Sorrell AM, Heo SY, Kang JH, et al. Production of human CD59-transgenic pigs by embryonic germ cell nuclear transfer. *Biochem Biophys Res Commun*. (2010) 400:667–72. doi: 10.1016/j.bbrc.2010.08.125

54. Uszewski KM. Control of the complement system. *Adv Immunol*. (1996) 61:201–83. doi: 10.1016/S0065-2776(08)60868-8

55. Ji YW, Ahn KS, Sorrell AM, Shin S, Shim H. Cytolytic assessment of hyperacute rejection and production of nuclear transfer embryos using hCD46-transgenic porcine embryonic germ cells. *Zygote*. (2009) 17:101–8. doi: 10.1017/S096719940800511X

56. Lee JH, Lee HJ, Nahm KM, Jeon HY, Hwang WS, Paik NW, et al. Effects of combined expression of human complement regulatory proteins and H-transferase on the inhibition of complement-mediated cytolysis in porcine embryonic fibroblasts. *Transplant Proc*. (2006) 38:1618–21. doi: 10.1016/j.transproceed.2006.02.129

57. Shim H. Isolation of pluripotent stem cells from cultured porcine primordial germ cells. *Biol Reproduction*. (1997) 47:1089–95. doi: 10.1095/biolreprod57.5.1089

58. Chen CG, Salvaris EJ, Romanella M, Aminian A, Pearse MJ. Transgenic expression of human alpha1,2-fucosyltransferase (H-transferase) prolongs mouse heart survival in an ex vivo model of xenograft rejection. *Transplantation*. (1998) 65:832. doi: 10.1097/00007890-199803270-00011

59. Rother RP, Rollins SA, Mojcik CF, Brodsky RA, Bell L. Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria. *Nat Biotechnol*. (2007) 25:1256. doi: 10.1038/nbt1344

60. Melis JPM, Strumane K, Ruuls SR, Beurskens FJ, Schuurman J, Parren PWHI. Complement in therapy and disease: Regulating the complement system with antibody-based therapeutics. *Mol Immunol*. (2015) 67:117–30. doi: 10.1016/j.molimm.2015.01.028

61. Woodruff TM, Nandakumar KS, Tedesco F. Inhibiting the C5-C5a receptor axis. *Mol Immunol*. (2011) 48:1631–42. doi: 10.1016/j.molimm.2011.04.014

62. Ricklin D, Lambris JD. New milestones ahead in complement-targeted therapy. *Semin Immunol*. (2016) 28(3):208–22. doi: 10.1016/j.smim.2016.06.001

63. Morgan BP, Harris CL. Complement, a target for therapy in inflammatory and degenerative diseases. *Nat Rev Drug Discovery*. (2015) 14(12):857–77. doi: 10.1038/nrd4657

64. Risitano AM, Marotta S. Therapeutic complement inhibition in complement-mediated hemolytic anemias: Past, present and future. *Semin Immunol*. (2016) 28(3):223–40. doi: 10.1016/j.smim.2016.05.001

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 17 April 2024
DOI 10.3389/fimmu.2024.1385022

OPEN ACCESS

EDITED BY
Lisha Mou,
Shenzhen Second People's Hospital, China

REVIEWED BY
Lingyu Bao,
National Institutes of Health (NIH), United States
Jin-Ming Zhang,
Capital Medical University, China

*CORRESPONDENCE
Yi Wang
w_yi2022@163.com
Zhengteng Yang
330560167@qq.com
Siyuan Song
si-yuan.song@bcm.edu

†These authors have contributed equally to this work

RECEIVED 11 February 2024
ACCEPTED 28 March 2024
PUBLISHED 17 April 2024

CITATION
Wang H, Wen L, Jiang F, Ren P, Yang Y, Song S, Yang Z and Wang Y (2024) A comprehensive review of advances in hepatocyte microencapsulation: selecting materials and preserving cell viability. *Front. Immunol.* 15:1385022. doi: 10.3389/fimmu.2024.1385022

A comprehensive review of advances in hepatocyte microencapsulation: selecting materials and preserving cell viability

Hailian Wang[1†], Lebin Wen[2†], Fengdi Jiang[3†], Pengyu Ren[3†], Yixin Yang[4†], Siyuan Song[5*], Zhengteng Yang[6*] and Yi Wang[7*]

[1]Clinical Immunology Translational Medicine Key Laboratory of Sichuan Province, Center of Organ Transplantation, Sichuan Academy of Medical Science and Sichuan Provincial People's Hospital, Chengdu, China, [2]Department of Thyroid, Sichuan Second Hospital of Traditional Chinese Medicine, Chengdu, Sichuan, China, [3]School of Medicine, University of Electronic Science and Technology of China, Chengdu, China, [4]Department of Clinical Medicine, The First Clinical Medical College of Norman Bethune University of Medical Sciences, Jilin, China, [5]Department of Neuroscience, Baylor College of Medicine, Houston, TX, United States, [6]Department of Pharmacy, Guangxi University of Chinese Medicine, Nanning, China, [7]Clinical Immunology Translational Medicine Key Laboratory of Sichuan Province, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, School of Medicine, University of Electronic Science and Technology of China, Chengdu, China

Liver failure represents a critical medical condition with a traditionally grim prognosis, where treatment options have been notably limited. Historically, liver transplantation has stood as the sole definitive cure, yet the stark disparity between the limited availability of liver donations and the high demand for such organs has significantly hampered its feasibility. This discrepancy has necessitated the exploration of hepatocyte transplantation as a temporary, supportive intervention. In light of this, our review delves into the burgeoning field of hepatocyte transplantation, with a focus on the latest advancements in maintaining hepatocyte function, co-microencapsulation techniques, xenogeneic hepatocyte transplantation, and the selection of materials for microencapsulation. Our examination of hepatocyte microencapsulation research highlights that, to date, most studies have been conducted *in vitro* or using liver failure mouse models, with a notable paucity of experiments on larger mammals. The functionality of microencapsulated hepatocytes is primarily inferred through indirect measures such as urea and albumin production and the rate of ammonia clearance. Furthermore, research on the mechanisms underlying hepatocyte co-microencapsulation remains limited, and the practicality of xenogeneic hepatocyte transplantation requires further validation. The potential of hepatocyte microencapsulation extends beyond the current scope of application, suggesting a promising horizon for liver failure treatment modalities. Innovations in encapsulation materials and techniques aim to enhance cell viability and function, indicating a need for comprehensive studies that bridge the gap between small-scale laboratory success and clinical applicability. Moreover, the integration of bioengineering and regenerative medicine offers novel pathways to refine hepatocyte transplantation, potentially overcoming the challenges of immune rejection and ensuring the long-term functionality of transplanted cells. In conclusion,

while hepatocyte microencapsulation and transplantation herald a new era in liver failure therapy, significant strides must be made to translate these experimental approaches into viable clinical solutions. Future research should aim to expand the experimental models to include larger mammals, thereby providing a clearer understanding of the clinical potential of these therapies. Additionally, a deeper exploration into the mechanisms of cell survival and function within microcapsules, alongside the development of innovative encapsulation materials, will be critical in advancing the field and offering new hope to patients with liver failure.

KEYWORDS

hepatocyte encapsulation, microencapsulation, coculture, xenotransplantation, alginate

1 Introduction

1.1 The evolution of liver transplantation

The liver, one of the human body's largest and most versatile organs, is incredible for being able to detoxificate, metabolize, and maintain complex interactions with other organs like the kidney and spleen. Despite its critical role, individuals suffering from liver-based metabolic disorders (LBMD), hepatocellular carcinoma, fulminant liver failure, and end-stage liver diseases often face limited treatment options. The landscape of liver disease treatment underwent a significant transformation in 1963 when Thomas E. Starzl and his team pioneered the first clinical trials of orthotopic liver transplantation (LT) in three patients (1). This groundbreaking procedure offered a new lease on life for patients with severe liver conditions, improving their lifespan and quality of life. The procedure's advantages include the liver's remarkable regenerative ability, which minimizes donor risk, and an overall increase in population survival rates. However, LT is not without its drawbacks, including surgical complications, high costs, and the requirement for lifelong immunosuppression. Above all, the chronic shortage of available organs has been a persistent hurdle, underscoring the need for more feasible treatment alternatives.

1.2 The advent and progress of hepatocyte transplantation

The growing discrepancy between the demand for liver transplants and the available supply, as highlighted in recent reports by the Organ Procurement and Transplantation Network (OPTN) and the Scientific Registry of Transplant Recipients (SRTR), underscores the urgent need for alternative liver tissue sources. Hepatocytes, or liver cells, possess distinct characteristics that make them particularly appealing for transplantation; they retain functional capabilities even when isolated, and cryopreserved hepatocytes can be rapidly deployed for urgent therapeutic needs. This realization sparked interest in the potential of hepatocyte transplantation (HT) as a viable alternative to LT for managing LBMD and acute liver failure (ALF). Although hepatocyte transplantation is limited by many obstacles in clinical practice, researchers are constantly working to overcome them. Tasks remains to solve include scarce supply of reliable and high-quality hepatocytes, sub-optimal survival and regeneration after hepatocyte transplantation with transient phenotype, and urgent need of more effective immunosuppressive protocols to reduce rejection (2). Demonstrated to functionally mimic the liver to a certain extent, especially in acute cases, HT presents several advantages over traditional LT. One donor liver could potentially benefit multiple patients, depending on the yield of viable cells obtained and the specific needs of each patient (3). HT offers a less invasive approach compared to LT, eliminating the need for major surgery. Additionally, the ability to repeat hepatocyte infusions and preserve cells for future use means that patients on the liver transplant waitlist can maintain some liver function until a suitable organ match is found. Furthermore, the lower costs, reduced risks, and fewer complications associated with HT have contributed to its growing popularity as a promising treatment alternative. The various encapsulation methods discussed herein are summarized in Figure 1, providing a concise overview of the innovative approaches in hepatocyte transplantation.

2 Advancements in encapsulation materials for hepatocyte transplantation

2.1 Factors and mechanism related

Hepatocyte Transplantation demonstrates several important factors. The location of encapsulated hepatocytes within recipients can influence their viability and functionality. Existing literature and

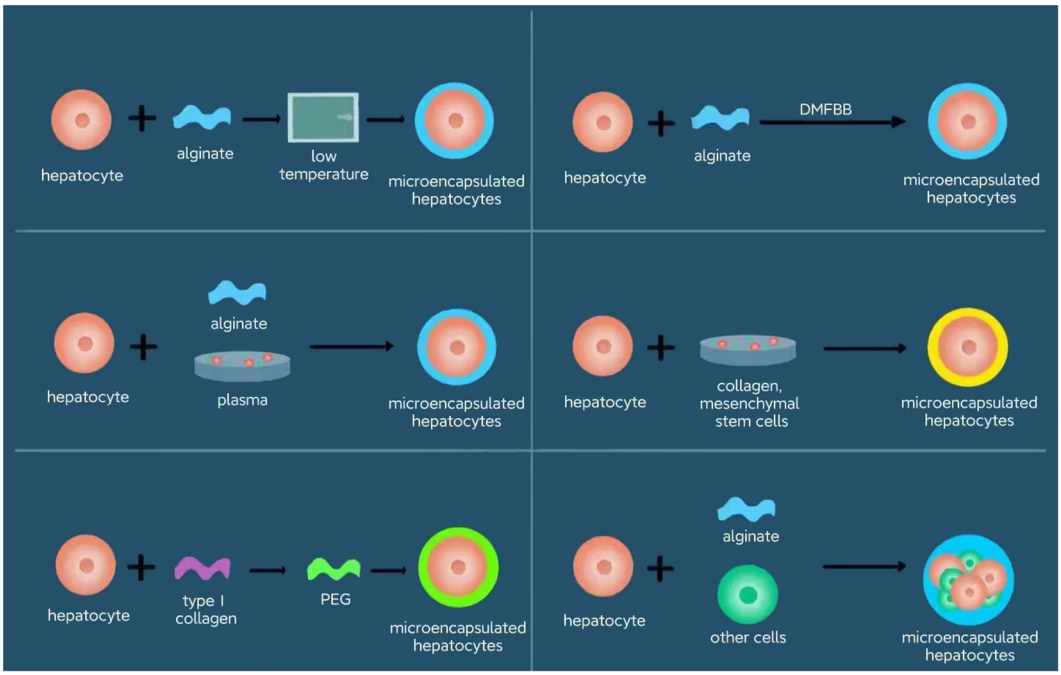


FIGURE 1
Encapsulation approaches for hepatocytes. The various encapsulation methods discussed herein are summarized, providing a concise overview of the innovative approaches in hepatocyte transplantation. The approaches include alginate plus low temperature, alginate plus plasma, type 1 collagen and PEG, alginate plus diversion-type microcapsule-suspension fluidized bed bioreactor (DMFBB), collagen plus mesenchymal stem cells, and alginate plus other cells.

experiments suggest that the implantation site plays a crucial role in the success of hepatocyte transplantation. For instance, encapsulated hepatocytes transplanted into the peritoneal cavity may benefit from the rich blood supply and immune-privileged status, which could enhance cell survival and function (4). The microenvironment at the transplantation site, including factors like oxygenation and nutrient availability, can significantly impact the encapsulated hepatocytes' ability to maintain their liver-specific functions.

The dose or number of transplanted hepatocytes is a critical factor affecting transplant efficiency and the therapeutic outcome, which together with the place of transplantation, will largely decide the condition of hepatocytes (5). Determining the optimal number of hepatocytes for transplantation remains a challenge and is subject to ongoing research. The efficiency of transplantation and subsequent liver function recovery is likely dose-dependent, requiring a balance between sufficient cell mass for therapeutic effect and the host's capacity to integrate and support the transplanted cells. Studies have suggested that a higher number of transplanted hepatocytes may improve the functional recovery in liver failure models, but this must be balanced against the risk of potential complications such as portal hypertension or embolization (6). Future research should aim to establish standardized protocols for dosing and to explore the mechanisms underlying dose-dependent effects on transplantation outcomes.

The detailed discussion on the underlying mechanisms of encapsulated hepatocytes is limited. Potential mechanisms behind the improved viability and function of encapsulated hepatocytes involve several aspects, including enhanced protection from immunological rejection, improved microenvironmental control within the capsules, and the supportive effects of co-encapsulated cells (7). For instance, encapsulation materials like alginate provide a semi-permeable barrier that can protect hepatocytes from the host's immune response while allowing the exchange of nutrients and metabolic products (8). Additionally, co-microencapsulation with supportive cell types, such as mesenchymal stem cells, may provide trophic support and promote a more physiologically relevant microenvironment that enhances hepatocyte function (9).

2.2 Addressing immune rejection challenges

Addressing challenges such as immune rejection after hepatocyte transplantation, encapsulation emerges as a straightforward, economical, and effective strategy. The critical aspects of this approach—material selection, encapsulation technique optimization, and culture environment adaptation—are key to successful hepatocyte encapsulation. The choice of encapsulation material is particularly crucial as it directly impacts the encapsulated hepatocytes' functionality and viability by influencing oxygen and nutrient transfer.

2.3 Alginate's role in hepatocyte microencapsulation

Alginate, a material favored for its biocompatibility, ease of gel formation, and unique physicochemical properties, stands out in

the realm of hepatocyte microencapsulation. Miranda et al. observed that alginate-encapsulated hepatocyte aggregates exhibited significantly enhanced albumin production, urea synthesis, and enzymatic activities such as 7-ethoxycoumarin O-deethylase and uridine diphosphate glucuronosyltransferase (UGT) compared to non-encapsulated controls (8). Notably, these encapsulated hepatocytes demonstrated improved functional outcomes when cultured in a bioreactor system, maintaining performance over three weeks.

In a novel exploration, Nhu-Mai et al. revealed that alginate hydrogel could shield human hepatoma-derived cells (Huh-7), the most commonly used cell line recently with high permissiveness, from Hepatitis C Virus (HCV) infection (10). This protective effect, dependent on the concentration and duration of culture, suggests alginate hydrogel's broader viral defense capability, irrespective of encapsulation.

2.4 Optimization and comparative studies

Further research by Lan et al. compared the survival and metabolic function of hepatocytes encapsulated in different alginate compositions, SLM100 and SLG100, demonstrating sustained viability, enzyme secretion, and antioxidant activity under 3D culture conditions, albeit with reduced proliferation rates (11). Saeed Azandeh et al. investigated the impact of alginate hydrogel concentrations on Human Wharton's Jelly-derived Mesenchymal Stem Cells (HWJ-MSCs) (12). They discovered that a 1.5% alginate concentration was more conducive to cell proliferation and urea production than a 2.5% concentration, highlighting the importance of finding the optimal alginate concentration for hepatocyte viability. Jitraruch et al. proposed an optimized protocol for producing alginate-encapsulated human hepatocytes, which demonstrated superior mechanical stability and ideal bead size for enhanced cell viability (13). Similarly, Pasqua et al. developed a technique for culturing hepatocytes in 1.5% alginate beads, facilitating the autonomous formation of spheroids with maintained liver functions over two weeks (14). Durkut et al. evaluated the viability and metabolism of primary rat hepatocytes encapsulated in various matrices and subjected to different cryopreservation conditions (15). Their findings indicated that cryopreservation in liquid nitrogen (LN2) best preserved hepatic functions and viability, with ACA-encapsulated hepatocytes maintaining nearly 90% of their metabolic activity post-thaw. These studies collectively underscore the significance of material selection and encapsulation conditions in enhancing the therapeutic potential of hepatocyte transplantation, paving the way for innovative approaches to liver disease treatment. The quest to mitigate immune rejection reactions post hepatocyte transplantation has steered research towards hepatocyte encapsulation as a viable, cost-effective solution. Key to this endeavor is the meticulous selection of encapsulation materials, microencapsulation methodologies, and the fine-tuning of the culture environment, all of which are pivotal for the successful encapsulation of hepatocytes.

2.5 Novel encapsulation materials and techniques

Stephanie H. Capone and associates explored various material combinations for cell microencapsulation, including alginate alone, alginate combined with type I collagen, with or without poly-L-lysine and alginate coatings (16). They discovered that incorporating collagen and polylysine enhanced the mechanical resilience of the beads but compromised vitamin B12 mass transfer kinetics. Alginate-collagen beads notably enhanced HepG2/C3A viability with increased metabolism rate. Upon subcutaneous implantation in mice, they also mitigated inflammation, spotlighting the crucial balance between mechanical strength, cell behavior, and biocompatibility. Subhas C. and his team innovated silk sericin–alginate–chitosan microcapsules, creating a sericin and alginate microbead core with a chitosan outer shell (17). These microcapsules, characterized by their spherical shape and glossy surface, demonstrated high cell viability and uniform encapsulation under confocal microscopy, indicating an optimized living microenvironment for the encapsulated cells.

2.6 Cell source considerations for clinical transplantation

A significant challenge in hepatocyte transplantation research has been sourcing cells that are both functional and safe for clinical use. Traditionally, hepatocellular carcinoma (HCC) cell lines such as HepG2, HepaRG, and HepG2/C3A have been extensively utilized in research due to their ease of propagation and maintenance. However, these cells are derived from liver cancers and, as such, are not suitable for clinical transplantation purposes. Their immortal nature, potential for uncontrolled proliferation, and inferior functionality compared to primary hepatocytes limit their applicability in therapeutic contexts. Recognizing these limitations, the field is increasingly turning towards human pluripotent stem cells (hPSCs) as a potential source of hepatocyte-like cells (HLCs) for transplantation. hPSCs, including both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), possess the capacity for unlimited self-renewal and the potential to differentiate into any cell type, including hepatocyte-like cells. This differentiation is achieved through the mimicking of liver development stages *in vitro*, leading to the generation of cells that exhibit key hepatocyte functions such as albumin secretion, urea production, and drug-metabolizing enzyme activity.

The use of hPSC-derived HLCs presents a promising avenue for overcoming the cell source limitation in hepatocyte transplantation. These cells could provide a renewable, ethically accessible, and potentially customizable source of hepatocytes for therapeutic applications. Furthermore, advancements in differentiation protocols and three-dimensional culture systems are enhancing the functional maturation of HLCs, bringing them closer to the functionality of primary human hepatocytes. However, challenges remain in the clinical application of hPSC-derived HLCs, including ensuring the efficiency and consistency of differentiation protocols,

the purity and safety of the cell populations (e.g., eliminating the risk of tumorigenicity), and the long-term functionality and integration of the transplanted cells *in vivo*. Addressing these challenges requires ongoing research and collaboration between stem cell biologists, tissue engineers, and clinical researchers. While HCC cell lines have provided valuable insights into liver biology and disease, the transition towards clinical transplantation necessitates the exploration of alternative cell sources like hPSC-derived HLCs. The promise of these cells in providing a viable, ethical, and functional source for hepatocyte transplantation underscores the importance of continued research and development in this exciting area of regenerative medicine.

2.7 Enhancing Liver-specific functions

Meng Tian et al. developed Galactosylated alginate (GA)–chitosan oligomer microcapsules, adjusting membrane porosity and thickness to balance mechanical stability and permeability (18). This selective permeability effectively transported human serum albumin while blocking immunoglobulin G, enhancing liver-specific functions within the microcapsules. In the study by Ying He et al., Cytodex 3 microcarriers formed the core of the microcapsules, enveloped by an alginic acid-chitosan-alginate (ACA) polyelectrolyte layer (19). Utilizing an aqueous two-phase emulsification technique, L02 cells on Cytodex-3 microcarriers were encased within a thin conformable layer, facilitating equitable transport of nutrients and wastes. These microcapsules consistently produced urea and human albumin *in vitro* and demonstrated the capability to stabilize serum markers in acetaminophen-damaged rats post-transplantation. Christian Siltanen et al. utilized a coaxial flow-focused droplet microfluidics approach to craft microcapsules with liquid cores and polyethylene glycol (PEG) shells (20). This encapsulation facilitated rapid aggregation of primary hepatocytes into dense globules, preserving liver function leaped from normally 1-2 days to 10 days. The technique also offered the flexibility to tailor the mechanical properties and permeability of the gel, making it adaptable for further experimental investigations. Shahla Khodabakhsh Aghdam et al. explored the incorporation of galactosylchitosan (GC) and collagen (COL) into alginate microcapsules, subsequently coated with chitosan to produce alginate-galactosylated chitosan-collagen/chitosan (AGCCol/C) microcapsules (21). This addition significantly influenced the hydrogels' physical properties, enhancing the proliferation of HepG2 cells, and up-regulating the expression of P450 and albumin mRNA, demonstrating improved biocompatibility. anhong Zhang et al. adopted a one-step spray method to fabricate microcapsules using hyaluronic acid (HA)/sodium alginate (SA) as the core and chitosan (CS) as the shell (22). This method not only ensured high viability of C3A cells *in vitro* but also enhanced urea and albumin synthesis, highlighting HA's role in promoting CYP450 gene expressions. Such advancements suggest a promising direction in hepatocyte microencapsulation techniques for liver transplantation applications. Further details and comparative analyses of these methodologies are summarized in Table 1, offering a comprehensive overview of the state-of-the-art in hepatocyte microencapsulation. Hyaluronic acid (HA) has been observed to enhance the expression of genes related to the cytochrome P450 family after a duration of three days. Utilizing an encapsulation technique, it was noted that the functionality of hepatocytes was markedly preserved, providing an improved habitat conducive to their survival. This method is seen as a potential option for the transplantation of hepatocyte microcapsules. Chan et al. explored an alternative approach by employing Double-emulsion (DE) droplets to generate microencapsulated homotypic or heterotypic hepatocyte spheroids within an alginate-collagen composite hydrogel, moving away from the sole use of alginate (42). Their microfluidics-based technique, which eliminates the necessity for spheroid loading and allows for the control over spheroid characteristics, has shown to enhance hepatocyte performance. This includes increased albumin and urea secretion, as well as improved cytochrome P450 activity. Moreover, hepatocyte function was further enhanced when co-cultured with endothelial progenitor cells at an optimal ratio of 5 to 1 in alginate-collagen. In contrast, Lee et al. experimented with hybrid hydrogels of varying stiffness to encapsulate HepaRG cells, either individually or with support cells. They utilized tissue engineering approaches to fabricate three-dimensional (3-D) liver models *in vitro* (43). When the elasticity of these 3D liver models was adjusted to closely match the range of 2.3-5.9 kPa, there was a notable increase in hepatic gene expression, albumin secretion, cytochrome p450-3A4 activity, and drug metabolism capabilities. This model also demonstrated the ability to extend the viability and functionality of hepatocytes over extended culture periods. Further contributing to this field, Cui and colleagues demonstrated that utilizing gelatin methacryloyl (GelMA) hydrogel as a base for constructing 3D lobule-like microtissues offers advantages for hepatocyte functionality (44). The GelMA hydrogel, shaped by a digital micromirror device (DMD)-based microfluidic channel, allows for the encapsulation of hepatocytes within micromodules featuring a central radial-type hole. After prolonged co-culture, hepatocytes encapsulated alongside fibroblasts showed an increase in albumin secretion and maintained over 90% cell survival rate. Chang et al. opted for volvox sphere microbeads to encapsulate hepatocytes, providing a dual-layer three-dimensional environment for the cells (45). This innovative approach contributes to the growing body of research focused on improving hepatocyte culture methods and transplantation strategies. Dynamic bioreactor cultures of AML12 hepatocytes together with rat mesenchymal stem cells (MSCs) demonstrated significant enhancements, with MSCs evolving into hepatocyte-like cells, doubling albumin (ALB) secretion, and increasing cytokeratin 18 expression by 2.5 times. In models of CCl4-induced liver damage in rats, encapsulation of MSCs and hepatocytes within volvox spheres markedly reduced AST and ALT levels, aiding liver repair and new tissue formation (46). Kim's investigation into a three-dimensional heparin-based hydrogel scaffold for hepatocyte culture revealed that such encapsulated hepatocytes maintained high-level functionality, including albumin and urea synthesis, for up to three weeks. The addition of hepatocyte growth factor (HGF) into the hydrogel further enhanced these synthesis processes (47).

TABLE 1 Co-encapsulated cells and their results in recent studies on co-encapsulated cells.

Year	Cell types	Donor	Recipient	Transplantation site	Results	Reference
2002	hepatocyte + bone marrow stem cell	Male Wistar rats (250-300 g)	Wistar male rats, 200-250 g	in vitro and in vivo (intraperitoneal)	improve the viability and feasibility of liver	(23)
2004	hepatocyte + bone marrow stem cell	Male SD rate (150-200 g)	not found	in vitro	improve the urea synthesis and albumin secretion activities	(24)
2004	hepatocytes +islets	Hepatocytes: Male Wistar rats (150 g); islets: Male Wistar rats (250 g)	Kunming mice (30-36 g)	in vitro and in vivo (intraperitoneal)	albumin level better maintained, BG sooner return to normal	(25)
2007	hepatocytes+human umbilical vein endothelial cells	hepatocytes: a subclone of rat hepatoma HIIE cells from Prof. K. Motojima, Meiji Pharmaceutical University, Tokyo, Japan; HUVECs: Toyobo Co., Ltd. (Osaka, Japan)	not found	in vitro	increase some genes expression caused by cell to cell communication	(26)
2003	hepatocyte+bone marrow stem cell	Male Wistar rats (200-250 g)	homozygous gunn rats, j/j, 200g	in vitro and in vivo (intraperitoneal)	maintain function in vitro and improve bio-ability in vivo	(27)
2008	rat hepatocytes + mouse fibroblast, NIH/3T3 cell	Male Wistar rat aged 5-8 weeks	not found	in vitro	promote cell growth and maintain all sorts of functions	(28)
2009	hepG2 +sertoli cells	not found	not found	in vitro	better function and bioactivity	(29)
2009	hepatocyte _bone marrow mesenchymal stem cell	SD rats (180-200 g)	SD rats (180-200 g)	peritoneum	improve albumin secretion and urea synthesis	(30)
2005	HepG2+sertoli cells	sertoli cell: Male Wistar rats, age 15-20 days; HepG2 cells: ECACC (Wiltshire, UK)	Young male Wistar rats	in vitro and in vivo (intraperitoneal)	protect cell with locally generated immuno-suppression	(31)
2010	rat hepatocyte_human fetal liver stromal cells	Wistar rats (160-180 g) and Balb/c mice (22-25 g)	Balb/c mice	in vitro and in vivo (peritoneum)	improve the survival of acute liver failure rat model and maintain function	(32)
2014	hepatocyte +adipose-derived stem cells	Female SD rats (180-200 g)	female SD rats (180-200 g)	in vitro and in vivo (peritoneum)	better functions and viability	(33)
2015	hepatocyte+human mesenchymal stem cells	human hepatocyte: reject or unuse for orthotopic liver transplantation or liver resections at King's College Hospital (London, UK); MSCs: human umbilical cord matrix	not found	in vitro	enhance urea synthesis and albumin secretion and improve the viability	(34)
2015	iPS-human+stem cells	iPS-H: laboratory of Dr. Stephen Duncan at the Medical College of Wisconsin; SCs: Dr. Howard Green at Harvard Medical School	C57BL/6 mice	in vitro and in vivo (intraperitoneal)	improve human albumin and α1-antitrypsin (A1AT)	(35)
2018	porcine hepatocyte +human mesenchymal stem cells	porcine hepatocyte: 10 kg pigs (M. Stirnimann, Apples, Switzerland); human MSCs: femoral head of the patients undergoing total hip replacement	not found	in vitro	day 3: possess albumin secretion and diazepam catabolism; day 4 and 8: better buo-activity and longer span of albumin secretion	(36)
2018	hepatocyte+mesenchymal stem cells	hepatocytes: reject or unuse for orthotopic liver transplantation; MSCs: Wharton's jelly (cords from C-sections)	Male SD rats 8-10 weeks (200-300 g)	in vitro and in vivo (intraperitoneal)	improve behavior and function	(37)

(Continued)

10.3389/fimmu.2024.1385022

TABLE 1 Continued

Year	Cell types	Donor	Recipient	Transplantation site	Results	Reference
2018	hepatocyte +human umbilical vein endothelial cells	hepatocyte: according to seglen; HUEVCs: ATCC	SD rats (180-220 g)	in vitro and in vivo (intraperitoneal)	maintain hepatocyte-specific function	(38)
2020	primary human hepatocytes +collagen fibroblasts	hepatocyte: 54-year-old female Caucasian, 16-year-old female AsianCollagen I: Corning Life Science, Tewksbury, MA, USA)	not found	in vitro	improve hepatic functions and gene expression	(39)
2020	hepatocytes+HNF4α-overexpressing human umbilical cord MSCs	hepatocyte: patients who undergoing partial hepatectomy or liver transplantation; UMSCs: Wharton's jelly of umbilical cord	Male C57BL/6 mice (8-10 weeks)	in vitro and in vivo (intraperitoneal)	produce therapeutic effect	(40)
2021	HepLPCs+HUVECs	according to previous study	NSG mice	in vitro and in vivo (intraperitoneal)	alleviate liver injury caused by CCL4	(41)

Gevaert et al. compared HepG2 cell encapsulation effects between galactosylated gelatin and Methacrylamide modified gelatin, finding that methacrylamide modification had little impact on viability, whereas galactosylated gelatin significantly enhanced specific gene expression over long-term culture (>21 days) (48). Lee and co-researchers discovered that hepatic function in primary human hepatocytes (PHHs) encapsulated within biodegradable hydrogel systems was best maintained with hydrogels of intermediate initial degradability, outperforming Matrigel in cytochrome P450 functional assays (49). Wang et al. introduced a one-step synthesis method for creating composite hydrogel capsules (CHCs) characterized by uniformity, biocompatibility, stability, and high-throughput capabilities, showing that hepatocytes encapsulated in CHCs exhibited enhanced viability, growth, and liver-specific functions (50). Tirella's study presented a protein/hydrogel formulation as a novel encapsulation choice, enhancing nutrient exchange and providing a 3D adhesive framework for cells. This study also included encapsulating ratiometric optical nanosensors within hepatocytes to monitor microenvironmental pH changes under stress, noting improved albumin secretion and urea production in encapsulated hepatocytes compared to controls (51). Khanal et al. developed a method for creating polymeric nanofiber-integrated alginate (PNA) hydrogel microcapsules using a Nano-in-micro (NIM) system, with PNA-10 showing optimal support for HepG2 cell growth and maintenance of liver-specific metabolic functions (52). Zheng and colleagues' research on self-bonding real-time shape-programmable microcapsules via photo-induced electrodeposition (PIED) of cell-laden alginate hydrogel found that pre-coating with fibroblasts led to robust assembly through fibroblast-ECM interactions, closely mimicking tissue morphogenesis. HepG2 cells encapsulated in these new microcapsules showed nearly double the albumin and urea secretion compared to non-fibroblast-coated encapsulations (53). Yu et al. experimented with microcapsules of various inner structures and deformability, finding that hepatocyte viability was consistent across different types, but cell activity was significantly reduced in capsules with lower deformability (54). Cui's work on spatially assembling gear-like microstructures from photo-crosslinkable poly (ethylene glycol) diacrylate (PEGDA) hydrogel, which co-encapsulated hepatocytes and fibroblasts, resulted in 3D lobule-like micro-architectures with high cell viability and proliferation, significantly enhancing albumin secretion and urea synthesis (55). Liu and colleagues' study on encapsulated rat liver (RLC-18) cells forming hepatic lobule-shaped microtissue (HLSM) reported superior hepatic-specific functions in these structures compared to normal cell spheroids after 14 days of culture in poly-L-lysine-alginate microcapsules (56). Moriyama et al. developed a method for producing hydrogel microbeads using an octa-thiolated PEG derivative (8-arm PEGSH), which maintained higher levels of specific functions including albumin secretion and urea production when HepG2 cells were encapsulated (57). Agarwal and team's application of decellularized Caprine liver ECM (CLECM) derived hydrogel for 2D and 3D hepatocyte cultures showed significantly enhanced functions, including albumin, urea, glycogen, and GAGs synthesis, and the formation of bile canaliculi-

like structures and better expression of mature hepatocyte markers compared to collagen coatings (58). Zhang et al. investigated the effects of 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) on Huh7 cells/C3A cells in both monolayer cultures and microspheres, noting significant improvements in protein levels and metabolic activities of major cytochrome P450 enzymes (59). Ravichandran et al. explored two methods to generate photocrosslinkable methacrylated liver extracellular matrix (LivMA) hydrogels, finding better cytocompatibility for encapsulated hepatocytes despite different mechanical properties (60). Zhang and colleagues compared hepatocytes encapsulated in lupeol liposomes and Gal-lupeol liposomes, with the latter showing higher cell-uptake and apoptotic efficiency in HepG2 cells, along with reduced expressions of AKT/mTOR-related proteins and markers *in vitro* and *in vivo*, demonstrating liver targeting effects in FVB mice (61). Leroux introduced a novel hybrid alginate microcapsule using an aqueous stable titania precursor (TiBALDH) and a cationic polyamine (PDDAC), leading to increased mechanical stability and maintained hepatocyte functions for up to 43 days (62). Sk's novel synthesis of photo-crosslinkable glycidyl methacrylate (GMA) functionalized gelatins (Gelatin-GMA) enhanced cell growth and cellular functions in Huh-7.5 cells encapsulated in 3D hydrogel scaffolds.

The cultivation environment's materials for microencapsulated cells significantly impact cell function preservation. Tostoes et al. discovered that liver-specific functions such as urea production, phase I drug metabolizing activity, and oxygen uptake in hepatocytes encapsulated within ultra-high viscous alginate spheroids were substantially improved under a continuous perfusion system compared to a traditional 50% medium change routine, tripling the performance. However, albumin output remained consistent across both feeding methods (63). Sofia P. et al. proposed a three-dimensional culture strategy for HepaRG cells in alginate microcapsules without dimethyl sulfoxide (DMSO), enhancing hepatocyte differentiation significantly over 2D cultures. This approach yielded a higher prevalence of hepatocyte-like over biliary-like cells, alongside improved protein secretion and ammonia detoxification, despite some variance in basal gene expression levels (64).

2.8 Challenges of translating encapsulation materials to clinical use

The diversity of materials used for the microencapsulation of hepatocytes presents a spectrum of opportunities and challenges for clinical translation. Among these, photo-crosslinkable methacrylate-based materials have gained attention for their versatility, tunability, and the precision with which they can be manipulated using light. However, translating such advanced materials into clinical use encompasses several hurdles, particularly concerning safety, efficacy, and regulatory approval.

2.8.1 Safety and biocompatibility

The primary concern with any biomaterial intended for clinical use is its safety and biocompatibility. Photo-crosslinkable methacrylates, while useful in creating stable and customizable encapsulation systems, must be rigorously tested to ensure they do not elicit adverse immune responses, cause inflammation, or release toxic degradation products within the body. Long-term biocompatibility studies are essential to assess the risks of using these materials in humans.

2.8.2 Degradation and clearance

Understanding the degradation behavior of methacrylate-based materials *in vivo* is crucial. The materials must degrade at a rate that is compatible with tissue healing and regeneration processes without causing obstruction or toxicity. Moreover, the degradation products must be safely metabolizable or excretable by the human body.

2.8.3 Regulatory approval

Gaining regulatory approval for new biomaterials can be a complex and lengthy process. Regulatory bodies, such as the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA), require comprehensive data on the manufacturing process, quality control, safety, and efficacy of the biomaterials. For photo-crosslinkable methacrylates and other novel encapsulation materials, demonstrating compliance with these requirements involves extensive preclinical and clinical testing.

2.8.4 Scalability and consistency

Translating laboratory-scale encapsulation processes to clinical-scale production presents challenges in ensuring scalability, consistency, and cost-effectiveness of the material synthesis and encapsulation procedures. Ensuring that the properties of photo-crosslinkable methacrylates remain consistent across batches is critical for maintaining the reliability of the encapsulation system.

2.8.5 Ethical and legal considerations

The use of synthetic materials in medicine also raises ethical and legal considerations, particularly regarding long-term outcomes and patient consent. Patients must be fully informed of the benefits and risks associated with the use of such materials in treatments. While photo-crosslinkable methacrylate-based materials and other innovative encapsulation strategies offer significant potential for enhancing hepatocyte transplantation therapies, their path to clinical application is paved with challenges. Addressing these requires a multidisciplinary effort, combining insights from materials science, biology, medicine, and regulatory science to ensure that the benefits of these advanced materials can be safely and effectively realized in clinical settings.

2.9 Future directions in hepatocyte encapsulation

Despite the promising attributes of embryonic stem cells, such as high proliferation, renewability, and pluripotency, their differentiation into hepatocytes faces technical challenges, requiring an optimal culture microenvironment. Tim Maguire et al. explored how an alginate-based microenvironment supports

cell viability, promotes differentiation, and enhances the functionality of embryonic stem cell-derived hepatocytes, demonstrating urea and albumin synthesis as key functional indicators. This finding suggests a viable alternative for sourcing human hepatocytes for transplantation (65). MacPherson and colleagues designed a 3D scaffold from a non-fibrous hydrogel, emphasizing mechanical properties and nanofiber morphology to enhance hepatocyte culture. Their findings showed stable maintenance of primary human hepatocytes' viability and functionality, outperforming Matrigel in cytochrome P450 assays (66). Chen et al. assessed the stability of various alginate-based microcapsules in plasma, finding that alginate-α-poly (L-lysine)-alginate (α-APA) microcapsules demonstrated superior stability over alginate-ϵ-poly (L-lysine)-alginate (ϵ-APA) and alginate–chitosan–alginate (ACA) capsules. The stability of these capsules was influenced by different factors, with heparin significantly affecting α-APA microcapsules, while HCO3- and H2PO4-/HPO42- impacted ϵ-APA and ACA capsules, respectively (67). Liu introduced a method for creating porous alginate beads (PABs) using an aqueous two-phase system (ATPS) emulsion technique, blending a cell/dextran (Dex) mixture with an alginate (Alg)/polyethylene glycol (PEG) mixture. This approach allowed for control over the pore size, improving cell activity, proliferation, and function of encapsulated HeLa and human liver cancer cells compared to those in general alginate beads (GABs) (68).

Shogo Nagata et al. devised a technique for encapsulating cells within nucleocapsid hydrogel microfibers, creating a fibrous 3D ECM-rich microenvironment suitable for *in vitro* liver tissue formation. Induced pluripotent stem cell-derived hepatocytes (iPSC-hepatocytes) in this setup displayed liver-specific characteristics, including albumin secretion and liver marker gene expression, and maintained structural stability, indicating their potential for liver failure rescue. Transplantation of these microfibers into immunodeficient mice showed human albumin presence in peripheral blood after three days, confirming their viability and function as implants (69). Further details are presented in Table 1.

3 Enhancing hepatocyte viability and functionality

Ensuring high activity levels in hepatocytes is crucial for their ability to substitute for failing liver functions. However, hepatocytes are notably delicate, with even minor damages potentially leading to cell death and loss of activity. For microencapsulated liver cells to fulfill their intended roles post-transplantation, preserving their viability and functionality becomes a critical concern.

3.1 Hepatocyte cryopreservation

The primary strategy for the long-term storage of microencapsulated hepatocytes is cryopreservation. The effectiveness of this method and the ability of hepatocytes to resume their functions upon thawing are areas of active research. Mai and colleagues demonstrated that primary rodent hepatocytes could retain their synthetic functions temporarily through encapsulation and cryopreservation as early as 2005 (70). Despite the influence of immortalization on certain hepatocyte-specific functions remains questionable, which is to remove the upper limit of cell proliferation set by telomerase by either gene reactivation or deactivation, their findings suggested that both naïve and genetically modified hepatocytes could maintain metabolic functions and improve survival rates in xenogeneic recipients with liver failure when encapsulated, cryopreserved, and then transplanted, marking a significant advancement in hepatocyte therapy.

A subsequent study by Hang focused on the functional recovery of hepatocytes after cryopreservation (71). Results showed that pre-incubation at 4°C for 12–24 hours, followed by encapsulation in alginate–poly-L-lysine–alginate microcapsules, significantly enhanced hepatocyte functions, including mRNA and protein levels, as well as albumin and urea secretion post-thawing. The morphology and albumin production of post-thaw hepatocytes closely matched those of directly cultured groups over several days, underscoring the reliability of cryopreservation for hepatocyte storage despite potential risks to cell viability and functionality.

Kilbride et al.'s research revealed that alginate-encapsulated HepG2 liver microcapsules subjected to cryopreservation and subsequent short-term exposure to temperatures below 10°C from 1 to 90 minutes showed increased cell proliferation during 7-16 days of culture (72). This method presents a more efficient and cost-effective approach to achieving higher cell densities (73).

Recent work by Jitraruch et al. identified a pan-caspase inhibitor (ZVAD) that enhances the ultrastructure of cryopreserved hepatocyte microbeads and reduces cell apoptosis when combined with other cytoprotectants such as des-feroxamine (DFO), and human serum albumin (HSA) in the cryopreservation process (74). This improved cryopreservation technique optimizes the use of hepatocytes for emergency applications.

3.2 Alternative strategies for sustaining hepatocyte function

The liver's metabolic capacity is immense, capable of processing nutrients as well as detoxifying substances and drugs. Koizumi et al. were the first to demonstrate that primary rat hepatocytes retain their drug metabolism and transport activities post-cryopreservation when encapsulated (75). Activities of a specific drug-metabolizing enzyme (CYP3A2) and drug transport for several substrates were maintained up to 120 days using a novel cryopreservation technique developed by the researchers.

Encapsulation aims to shield hepatocytes from the host's immune system, yet the release of bioactive molecules from hepatocytes can potentially trigger immune responses (31). The extent of this reaction depends on the encapsulation material's permeability and the host's sensitivity. In animal studies, Baldini et al. showed that long-term cryopreserved encapsulated porcine hepatocytes maintained significant activity and viability when

transplanted into rats without immunosuppression (4). Although the ultrastructure and morphological activity of encapsulated hepatocytes were maintained post-explant, albumin synthesis was adversely affected, indicating a need for further improvements in maintaining bio-activity post-transplantation. Maximizing the use of donor livers involves isolating hepatocytes for propagation, making the retention of their functional capabilities post-isolation critically important.

Another investigation intended to test efficacy of bioartificial liver device in 2014. In this investigation, the evaluation of Alginate-chitosan microencapsulated hepatocytes' bioactivity was based on several metrics: cell proliferation, efficiency in ammonia detoxification, albumin production, and the rate of diazepam metabolism. The findings highlighted that, with the exception of cell proliferation which remained constant, immortalized human hepatocytes (HepLL) groups demonstrated superior performance in ammonia detoxification, albumin production, and diazepam metabolism compared to the HepG2 groups across all time points (29). Additionally, the viability of hepatocytes in spinner cultures showed variability over time, with day 10 marking the peak of cell growth, metabolic activity, and functionality.

Yamada et al. introduced a culture and encapsulation technique utilizing a Thermo-reversible gelation polymer (TGP), which transitions from solid to liquid states with temperature changes. This study revealed that hepatocytes encapsulated in TGP maintained over 70% viability after being cryopreserved in liquid nitrogen. Post-transplantation into the rat spleen, these hepatocytes were capable of performing liver-specific functions and secreting albumin (23).

Li and colleagues devised a method for hepatocyte encapsulation that involved micropatterning on collagen I to direct cell–cell interactions in two dimensions, followed by the formation of stable aggregates through collagenase digestion for three-dimensional encapsulation in polyethylene glycol (PEG) diacrylate. This configuration preserved the encapsulated hepatocytes' specific functions for up to 50 days (27).

Lu and associates developed an innovative diversion-type microcapsule-suspension fluidized bed bioreactor (DMFBB), offering several enhancements over the traditional fluidized bed bioreactor (FBB), especially under conditions of high perfusion velocity. The research noted a significant reduction in the void volume of alginate/chitosan microcapsules and lower damage rates during the fluidization process in the DMFBB. It was observed that encapsulated C3A cells exhibited higher survival rates and activities of CYP1A2 and CYP3A4 in the DMFBB, though improvements in albumin and urea synthesis were modest. Additionally, there was a notable upregulation in the transcription levels of various CYP450-related genes and an albumin-related gene in C3A cells within the DMFBB (24).

3.3 Enhancing hepatocyte function through co-microencapsulation

As hepatocyte transplantation emerges as a viable option for treating acute liver failure, the sustained activity and functionality of individual hepatocytes until the point of liver transplantation remain challenges. Recognizing that the liver comprises not only hepatocytes but also non-parenchymal derivatives, which provide essential structural, biochemical support, and nutrients, underscores the pivotal role mesenchymal cells play *in vivo*. Their presence is crucial for supporting the physiological activities of hepatocytes. This understanding has spurred interest in the co-culture and co-microencapsulation of hepatocytes with various mesenchymal cells to augment hepatocyte survival and functionality, particularly for cell transplantation applications primarily investigated in animal models. While co-culture and co-microencapsulation differ significantly, insights from hepatocyte co-culture studies offer valuable perspectives for advancing co-microencapsulation strategies. Initial investigations by Rahman et al. demonstrated the protective effects of co-encapsulating HepG2 cells with Sertoli cells in animal models of acute hepatic failure (AHF), achieving localized immunosuppression and enhancing HepG2 cell survival post-intraperitoneal injection in rats (76). This approach suggested a novel strategy for cell transplantation, potentially reducing rejection risks by locally generating immunosuppressive environments.

Zheng et al. conducted further research to ascertain the efficacy of co-microencapsulating Sertoli cells with HepG2 cells in a rat model, aiming to establish a method of local immunosuppression facilitated by the unique immunoprivileged nature of Sertoli cells (30). Their findings indicated that such co-microencapsulation could enhance the function and bioactivity of hepatocytes in models of acute liver failure, offering a more effective solution than either mixed or solely microencapsulated hepatocytes and Sertoli cells.

Moreover, Liu and collaborators explored the potential of co-encapsulating hepatocytes with bone marrow stem cells using a novel two-step cell encapsulation technique. This method proved to enhance hepatocyte viability and support liver function in models of acute liver failure (34). Compared to traditional single-step encapsulation, this innovative approach resulted in extended hepatocyte viability beyond four months post-transplantation, with a noticeable reduction in host reaction and improved hepatocyte function due to the synergistic effects of co-encapsulation with bone marrow cells. Further investigations confirmed the superior viability and functionality of this co-encapsulation strategy both *in vitro* and *in vivo*, demonstrating its capacity to ameliorate conditions like hyperbilirubinemia in Gunn rats post-transplantation (36).

Isoda et al. identified bone marrow stromal cells (BMSCs) as another promising candidate for hepatocyte co-culture, showing significant support for differentiated hepatocyte functions, notably in enhancing urea synthesis and albumin secretion (37). Their sandwich-like co-culture model, comprising a monolayer of BMSCs, a semi-permeable membrane, and freshly isolated hepatocytes, revealed the critical role of interleukin-6 in maintaining these key hepatocyte functions.

The exploration of mesenchymal stem cells (MSCs), known for their limited self-renewal while multidirectional differentiation capabilities, has become a focal point of recent research (40). The minimal ethical and legal hurdles associated with MSCs, coupled with their easy extraction from various sources including umbilical cord, endometrial polyps, menstrual blood, bone marrow, and

adipose tissue, make them a compelling option for experiments in hepatocyte co-encapsulation. Shi and colleagues highlighted the potential of bone marrow mesenchymal stem cells (BM-MSCs) in enhancing hepatocyte functionality when co-encapsulated, observing significant improvements in hepatocyte survival, liver function, and cellular changes post-transplantation using immunofluorescence microscopy. This study illustrated not only an increase in the specific functions of hepatocytes, such as albumin secretion and urea synthesis, but also an enhancement in cell cycle progression *in vitro*. Furthermore, hepatocyte transplantation strategies incorporating co-encapsulation demonstrated enhanced viability and bioactivity in rats models of acute liver failure, with MSCs potentially differentiating into hepatocyte-like cells and assuming liver metabolic functions (25).

Fitzpatrick et al. explored the advantages of coculturing human MSCs with hepatocytes, observing significant benefits in hepatocyte functions and viability when in direct or indirect contact with MSCs (26). This interaction notably increased albumin and urea production, with peak effects observed around day 15 for albumin. The study confirmed that coculturing hepatocytes with MSCs could enhance hepatocyte viability by up to 16%, suggesting a promising approach for cell transplantation.

Yang et al. developed a tissue engineering-based platform using cell-laden microbeads in a 3D printed tubular perfusion bioreactor, finding that co-encapsulation of human hepatocytes with collagen and MSCs resulted in improved cell activity and maintenance of parenchymal cell functions for up to 30 days (77). This setup facilitated better oxygen and medium diffusion, vital for sustaining cell vitality. Montanari and colleagues focused on the coculture and co-microencapsulation of porcine hepatocytes with human MSCs, identifying a beneficial role of MSCs in enhancing hepatocyte bioactivity and function (28). Their findings indicated that while hepatocyte viability may initially decrease, coencapsulation with MSCs led to sustained albumin secretion and diazepam metabolism, underlining the positive impact of MSCs on hepatocyte functionality.

Iansante et al. established a high-throughput system for cell encapsulation research, enabling the comparison of various conditions such as cell numbers, combinations, and alginate modifications (39). Their platform revealed that MSCs could notably improve the behavior and function of hepatocyte microcapsules. This enhancement was further validated through low-throughput analysis, underscoring the promising role of MSCs in boosting hepatocyte function.

Kong and colleagues demonstrated the therapeutic effects of co-encapsulating hepatocytes with HNF4α-overexpressing human umbilical cord MSCs (HNF4α-UMSCs) in models of acute liver failure (33). Their research showed that HNF4α-UMSCs could significantly enhance hepatocyte microbead functions and accelerate M2 macrophage polarization, potentially reducing the inflammatory response through the paracrine factor HB-EGF secreted by HNF4α-UMSCs. This study not only confirmed the functional benefits of co-encapsulation but also highlighted the underlying mechanisms contributing to improved outcomes in acute liver failure treatment.

Gao et al. explored co-encapsulation of hepatocytes with islets to create a bioartificial liver support system, showing a marked improvement in survival rates and biochemical parameters in ALF mice models (32). Takayama et al. discovered that co-culturing hepatocytes with human umbilical vein endothelial cells (HUVECs) enhances cellular functions, attributing this improvement to increased expression of certain genes linked to cell-to-cell communication (38). This insight into gene expression dynamics underlines the potential of co-culture systems in advancing liver tissue engineering.

Kim and colleagues developed a co-culture system based on cell sheets, demonstrating sustained albumin secretion and enhanced expression of hepatocyte-specific genes, thus significantly preserving hepatocyte functions (41). Nishikawa et al. showed that co-cultivating rat hepatocytes with NIH/3T3 fibroblasts on collagen-immobilized PDMS membranes enhances growth and function, notably albumin secretion, by providing ample oxygen (35).

Kukla et al. found that co-encapsulation of primary human hepatocytes with supportive fibroblasts significantly improves hepatic functions and gene expression, highlighting the benefits of incorporating 3T3-J2 murine embryonic fibroblasts or primary human hepatic stellate cells (HSCs) (78). Zhang et al.'s research on co-encapsulating hepatocytes with adipose-derived stem cells (ADSCs) demonstrated dramatic advantages in enhancing hepatocyte functions and viability, suggesting a potent cell-based therapy for liver failure (79). Teng and colleagues introduced a strategy employing rat hepatocytes and human fetal liver stromal cells (hFLSCs) for acute liver failure treatment, showing that the co-encapsulated approach significantly improves survival and hepatic function, partly due to the release of basic fibroblast growth factor (bFGF) (80). Qiu et al. confirmed the efficacy of co-encapsulating hepatocytes with HUVECs in treating fulminant hepatic failure (FHF), observing improved biochemical parameters and reduced mortality in rat models (81).

Liu's study on co-encapsulating human hepatocyte-derived liver progenitor-like cells (HepLPCs) with HUVECs highlighted the potential of this approach in ameliorating liver injury in mice, facilitated by the secretion of glial cell line-derived neurotrophic factor (GDNF) from HUVECs (82). Song et al. demonstrated that co-encapsulation of human induced pluripotent stem cell-derived hepatocyte-like cells with stromal cells in hydrogel capsules maintains human albumin and α1-antitrypsin levels effectively in mouse sera, mirroring the performance of primary hepatocyte aggregates (83). Most recently, Xiang Yuan's research on proliferating human hepatocytes (ProliHHs) revealed that Encapsulated ProliHHs could be engineered, intraperitoneally transplanted to those liver-failure animals, causing liver functions to reinforce though alleviated hyperammonemia and hypoglycemia, leading to less severe post-hepatectomy liver failure (PHLF) with minimal inflammatory response, adverse effects or tumorigenic (84).

These findings collectively underscore the vast potential of co-encapsulation strategies in enhancing hepatocyte functionality and viability, offering new avenues for liver failure treatment and tissue engineering. Further details are summarized in Table 2.

TABLE 2 The selection of materials for hepatocyte microencapsulation and its influence.

Year	Material	Cell type	Donor	Site	Result	Reference
2010	heparin-based hydrogel	hepatocytes	female adult Lewis rats (125-200 g)	in vitro	maintain albumin and urea synthesis	(21)
2013	alginate+type I collagen +poly-L-lysin +alginate		HepG2/C3a HCC cells	female C57BL/6 mice (7 weeks old)	in vitro and in vivo	(10)
2014	silk sericin-alginate-chitosam microcapsules	HepG2	culture in DMEM	in vitro	high cell viability and uniform cell encapsulation distribution, increase glucose consumption urea secretion rate and albumin content	(11)
2014	galactosylated alginnate (GA)-chitosan oligomer microcapsule	normal human hepatocytes, L02	CAS	in vitro	transport albumin block IgG, enhance liver function, high viability and proliferation of human hepatocytes	(12)
2014	glalctosylated gelatin/methacrylamide modified gelatin	HepG2	Prof. M. Bracke (Ghent University	in vitro	methacrylamide modified gelatin: no influence on hepatocyte viability galactosylated gelatin: improve specific gene expression >21 days	(22)
2015	protein/hydrogel	HepG2	ATCC, passage 83	in vitro	increase albumin secretion and urea production	(44)
2016	alginage-collagen composite hydrogel	Fresh primary SD rat hepatocytes	fresh hepatocyte	in vitro	increase albumin secretion, urea secretion and cytochrome P450 activity	(17)
2016	SiO2, polu(sodium-p-styrenesulfonate)(PSS), CaCO3, porous CaCO3 spheres	SMCs cells, HepG2 Cells and Ecs cells	Cell bank of CAS	in vitro	no difference in viability between three microbeads, reduce cell activity in microcapsules with lower defonmability	(47)
2016	octa-thiolated PEG derivative (8-arm PEGSH), horseradish peroxidase, a small phenolic compound (Glycyl-L-tyrosine)	HepG2 cells	National Bio-Resource Project of MEXT, Japan	in vitro	maintain albumin secretion and urea production	(50)
2017	Cytodex 3 microcarrier+alginic acid-chitosan-alginate (ACA) polyelectrolyte layer	L02 cells	GE Healthcare, UK	in vitro and in vivo (SD rats, 8 weeks, intraperitoneal)	in vivo: maintain the serum levels of total BiL, ALT and albumin in acetaminophen	(13)
2017	liquid cores+poluethylene glycol (PEG)	primary hepatocytes of female Lweis rats (125-200 g)	female Lewis rats (125-200 g) from Charles River laboratories (Boston, MA)	in vitro	allow adjustment of the mechanical properties and diffusion of the gel, maintain liver function for more than 10 days	(14)
2017	hybrid hydrogel	undifferentiated hepaRG cells	HPR101, Biopredic International, Saint Gregoire, France	in vitro	enhance hepatic gene expression, albumin secretion, cytochrome P450-3A4 activity, and drug metabolism	(18)
2017	volvox	MSCs and AML12 cells	the femurs of 3 week old SD rats	in vitro and in vivo (male 6-week-old SD rats implantation into liver)	in vitro: MSCs differentiate into hepatocyte like cell, increase albumin secretion, increase cytokeratin 18 expression; in vivo: reduce AST and ALT levels, improve repair and formation	(20)
2017	poly-L-lysine-alginate	rat liver (RLC-18) cells	culture with DMEM	in vitro	hepatic-specific function higher than normal cell spheroids	(49)

(Continued)

10.3389/fimmu.2024.1385022

TABLE 2 Continued

Year	Material	Cell type	Donor	Site	Result	Reference
2017	2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester	Huh7 and C3A cell	CRL-10741, ATCC	in vitro	monolayer cultures: improve protein levels and the metabolic activities and the SYP450 enzymes, SYP1A1, CYP1A2, CYP3A4 and CYP1B1; on mimcrospheres: increase the protein levels and some protein activities	(52)
2018	photo-crosslinking poly)ethylene glycol) diacrylate (PEGDA) hydrogel	HepG2 and NIH/3T3 cells	ATCC	in vitro	exhibit high cell viability, proliferatio and spreading, increase albumin secretion and urea synthesis	(48)
2018	decellularized caprine liver ECM (CLECM) derived hydrogel	HepG2	NCCS, Pune, India	in vitro	for 2D hepatocyte culture: increase albumin, urea,glycogen and GAG synthesis; for 3D culture: bile canaliculi-like structure, better expression of mature hepatocyte markers	(51)
2018	hybrid alginate+aqueous stable titania precursor +cationic poluamine	HepG2 cells	culture in DMEM	in vitro	microcapsule exhibit increased mechanical stability, maintain viability, oxygen consumption, and albumin secretion for 43 days	(55)
2019	gelatin methacryloyl hydrogel	HepG2 and NIH/3T3 cells	ATCC	in vitro	co-encapsulated with fibroblasts indicate increasing albumin secretion and over 90% cell survival	(19)
2019	polumeric nanofibreintegrated alginate	HepG2 cells	ATCC	in vitro	PNA-10 was suitable for HepG2 growth, 3D PNA-10 microcapsule maintain survival and liver-specific metabolic functions	(45)
2019	pre-coated with fibroblasts into alginate hydrogel	NIH/3T3 cells, HepG2 cells	ATCC	in vitro	increase albumin and urea secretion nearly 2 times	(46)
2020	alginate-galatosylated chitosan-collagen/chitosan	HepG2 cells	Pasture Institute, Iran	in vitro	promote the proliferation and secretion of albumin and urea, up-regulate the expression of P450 and albumin mRNA	(15)
2020	PEG/HA semi-IPN hydrogels	immortalized human hepatic sinusoidal EC-SV40 cells +fibroblasts	T0056, Applied Biological Materials, Inc., Richmond, BC, Canada	in vitro	maintain function outperform matrigel in CYP450 functional assays	(42)
2020	composite hydrogel	human liver cell, human iPSCs	cell bank of CAS	in vitro	better viability, growth, liver specific function including urea synthesis and albumin secretion	(43)
2021	hyaluronic acid (HA)/sodium alginate (SA)+chitosan (CS)	C3A cells	ATCC	in vitro	high viability, HA increases synthesis of urea and albumin, and the activity of CYP1A2, CYP3A4 and CYP450	(16)
2021	photocrosslinkable methacrylated liver extracellular matrix (LivMA) hydrogels	immortalized human hepataocytes cells	professor Didier Trono from the Ecole Polutechnique Federale de Lausanne, EPFL	in vitro	indicate better cytocompatibility	(53)
2021	Lupeol liposomes/Gal-lupeol liposomes modified with Gal-PEG-DSPE	HepG2	not found	in vitro and in vivo (FVB/N mice, high pressure tail vein transfection)	Gal0-lupeol-liposome: highest cell-uptake efficiency and higher apoptotic efficiency, Gal-lupeol-liposome: reduce expression of Akt/mTOR-related proteins in vitro, AFP, GPC3, and EpCAM mRNA expression	(54)
2021	gelatin derivatives-photo-crosslinkable glycidyl methacrylate (GMA) functionallized gelatins	Huh-7.5 cells	RIKEN (VA, Japan)	in vitro	enhance cell growth, improve differentiation, viability and proliferation	(56)

10.3389/fimmu.2024.1385022

4 The impact of xenotransplantation

With hepatocyte transplantation emerging as a notable strategy for addressing acute liver diseases, the scarcity of human liver donations has prompted the exploration of xenotransplantation. This approach, involving the transplantation of hepatocytes from other species, offers a potential solution to the shortage of allogeneic hepatocytes. However, numerous challenges, including immunological rejection and the potential for anaphylactic reactions to xenoproteins, necessitate further investigation. Studies have shown that encapsulation can enhance the survival and function of both fresh and cryopreserved porcine hepatocytes in models of fulminant liver failure (85). These studies revealed two critical phases: an *in vitro* decline in metabolic functions over a week post-transplantation and an *in vivo* extension of survival rates and maintenance of metabolic functions in encapsulated hepatocytes compared to non-encapsulated controls. Sgrio et al. discovered that encapsulated human hepatocytes, immortalized to stabilize metabolic functions, could substantially support metabolism and mitigate liver regeneration inhibition in acute liver failure models by reducing inflammatory stress (86). This dual approach highlights the need for distinct research focuses on metabolic function and regeneration in acute liver failure studies. Furthermore, encapsulated transplantation was found to significantly reduce cytokine levels, illustrating a decrease in inflammatory stress and a restraint on the regeneration of remaining hepatocytes (87).

Investigations into porcine hepatocytes as a xenotransplantation source have identified potential limitations, including safety concerns related to porcine endogenous retroviruses. Despite these challenges, studies have demonstrated therapeutic effects of encapsulated porcine hepatocytes in rodent and non-human primate models of fulminant liver failure (88). The use of neonatal pig re-aggregated liver cells (NPRLCs) has shown promise in improving survival rates and metabolic function in acute liver failure models, suggesting an alternative to alleviate the human hepatocyte shortage (89).

Machaidze et al. explored the transplantation of encapsulated miniature swine hepatocytes in baboons with fulminant liver failure, revealing a temporary support to liver metabolism and a restoration of normal liver functions in the majority of the treated animals (82). This indicates a viable method for large mammal xenotransplantation. Varaa et al. examined the effects of umbilical cord stem cells (UCSCs) and UCSC-derived hepatocyte-like cells (HLCs) encapsulated in high mannuronic alginate scaffolds on acute liver failure models, showcasing significant improvements in liver function markers (90).

Xenotransplantation research suggests that while xenohepatocytes offer a readily available solution to hepatocyte scarcity, the immunorejection challenge remains significant. Therefore, less immunogenic transgenic pigs and innovative cell encapsulation techniques are being considered as future research directions to address these hurdles (91). This overview underscores the complexities and potential of xenotransplantation in hepatocyte therapy, emphasizing the need for ongoing investigation into improving viability and functionality through advanced encapsulation methods and genetic modifications. Details on these studies are summarized in Table 2.

5 Clinical applications and future prospects

Although encapsulated hepatocyte technology has demonstrated promising results *in vitro* and in animal models, its transition to clinical applications presents a horizon ripe with potential. The clinical implications of hepatocyte encapsulation span several critical areas in liver failure treatment and regenerative medicine.

One primary application envisaged for encapsulated hepatocytes is in the development of a bioartificial liver device (BAL). Such devices aim to provide temporary liver support for patients with acute liver failure, bridging the gap to liver regeneration or transplantation. Encapsulated hepatocytes within BALs offer a biocompatible and immunoprotected environment, which could enhance cell function and longevity, thus improving the therapeutic efficacy of these devices. Moreover, the potential for allogeneic or xenogeneic cell transplantation without the need for lifelong immunosuppression could revolutionize the treatment landscape for liver diseases. The microencapsulation technique acts as a barrier to immune cells while allowing the exchange of nutrients and metabolic waste, making it a promising approach for cell transplantation therapies.

Clinical trials exploring the efficacy and safety of encapsulated hepatocyte transplantation are crucial next steps. Such studies will help determine the optimal cell sources, encapsulation materials, and transplantation protocols. Additionally, understanding the long-term outcomes of these interventions, including the risk of potential complications and the durability of treatment effects, is essential. Furthermore, integrating advances in biomaterials and stem cell technology could enhance the clinical applicability of hepatocyte encapsulation. For instance, the use of stem cell-derived hepatocytes for encapsulation could overcome the limitations associated with donor cell availability. Innovations in encapsulation materials that mimic the liver extracellular matrix could further support hepatocyte function and integration post-transplantation. In conclusion, while encapsulated hepatocytes herald a promising frontier in liver failure therapy, significant efforts in clinical research and technology development are necessary to translate these experimental approaches into viable clinical solutions. The progression of encapsulated hepatocyte technology into clinical trials and ultimately clinical practice will require multidisciplinary collaboration among scientists, clinicians, and regulatory bodies to ensure safety, efficacy, and patient accessibility.

6 Summary and future directions

This review has delved into the advancements in hepatocyte encapsulation research, emphasizing the strides made in preserving

hepatocyte viability, the innovative approach of co-microencapsulation, the exploration of xenotransplantation, and the development of novel encapsulation materials. Hepatocyte transplantation represents a promising avenue for mitigating the immune response challenges and addressing the scarcity of liver donations, offering a beacon of hope for numerous patients. The preservation of hepatocyte activity prior to transplantation is predominantly managed through encapsulation techniques, cryopreservation, enzyme inhibitors, and immunosuppressive agents. Future investigations could enhance these methods by fine-tuning temperature controls and elucidating the roles of specific enzymes in the longevity and functionality of transplanted hepatocytes.

The strategy of co-encapsulating hepatocytes with various cell types, such as Sertoli cells, bone marrow mesenchymal stem cells (MSCs), fibroblasts, adipose-derived stem cells, and human umbilical vein endothelial cells (HUVECs), has shown to enhance the survival and functionality of hepatocyte transplants. However, the underlying mechanisms of these co-encapsulation benefits remain to be fully understood and warrant further exploration. Xenotransplantation has emerged as a viable strategy to broaden the donor pool for hepatocyte transplantation. Research has predominantly focused on encapsulating and transplanting porcine or human liver cells into models of liver failure. These preclinical endeavors have demonstrated notable improvements in the functionality of transplanted liver cells. Advancements in the materials used for hepatocyte microencapsulation have also been significant, ranging from the optimization of traditional substances like alginate to the introduction of novel materials and structures for hepatocyte microcapsules, as well as refining the pre-encapsulation cell culture environments.

Last but not least, some of the accomplishments in animal and human-cell based studies are still in need of a more cautious attitude towards clinical achievement in reality. Many experiments take advantage of the marker serum albumin to check cell viability, which indeed has a long half-life does not fit in a lot. Investigation needs to be done on what actually happens to the liver cells from stem cells to its ultimate form due to their unique ability of regeneration, possibly using the Flow Cytometry techniques (92).

Looking ahead, hepatocyte microencapsulation research should aim to diversify the sources of transplantable cells, minimize the need for immunosuppression, and enhance the survival and functionality of transplanted hepatocytes. Such efforts will not only extend the applicability and safety of hepatocyte microencapsulation techniques but will also provide greater insights into liver cell biology and transplantation methodologies, ultimately benefiting a wider spectrum of patients with liver failure.

Author contributions

HW: Writing – original draft. FJ: Writing – original draft. PR: Writing – original draft. YY: Writing – review & editing. SS: Writing – review & editing. ZY: Funding acquisition, Writing – review & editing. YW: Conceptualization, Funding acquisition, Project administration, Writing – review & editing. LW: Writing – original draft.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was supported by the National Natural Science Foundation of China (81802504), grants from the Sichuan Science and Technology Bureau (2021YFH0384 and 2023YFH0010), and a grant from the Guangxi Science and Technology Base and Talent Special Project (AD23023011).

Conflict of interest

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

Publisher's note

References

1. Nguyen MP, Jain V, Iansante V, Mitry RR, Filippi C, Dhawan A. Clinical application of hepatocyte transplantation: current status, applicability, limitations, and future outlook. *Expert Rev Gastroenterol Hepatol.* (2020) 14:185–96. doi: 10.1080/17474124.2020.1733975

2. Sun Z, Yuan X, Wu J, Wang C, Zhang K, Zhang L, et al. Hepatocyte transplantation: The progress and the challenges. *Hepatol Commun.* (2023) 7(10): e0266. doi: 10.1097/HC9.0000000000000266

3. Hansel MC, Gramignoli R, Skvorak KJ, Dorko K, Marongiu F, Blake W, et al. The history and use of human hepatocytes for the treatment of liver diseases: the first 100 patients. *Curr Protoc Toxicol.* (2014) 62:14 12 1–23. doi: 10.1002/0471140856.tx1412s62

4. Baldini E, Cursio R, Sousa De G, Margara A, Honiger J, Saint-Paul M-C, et al. Peritoneal implantation of cryopreserved encapsulated porcine hepatocytes in rats without immunosuppression: viability and function. *Transplant Proc.* (2008) 40:2049–52. doi: 10.1016/j.transproceed.2008.05.038

5. Canaple L, Rehor A, Hunkeler D. Improving cell encapsulation through size control. *J Biomater Sci Polym Ed.* (2002) 13:783–96. doi: 10.1163/156856202760197410

6. Mao SA, Glorioso JM, Nyberg SL. Liver regeneration. *Transl Res.* (2014) 163:352–62. doi: 10.1016/j.trsl.2014.01.005

7. Fitzpatrick E, Filippi C, Jagadisan B, Shivapatham D, Anand H, Lyne M, et al. Intraperitoneal transplant of Hepatocytes co-Encapsulated with mesenchymal stromal cells in modified alginate microbeads for the treatment of acute Liver failure in

Pediatric patients (HELP)-An open-label, single-arm Simon's two stage phase 1 study protocol. *PloS One*. (2023) 18:e0288185. doi: 10.1371/journal.pone.0288185

8. Miranda JP, Rodrigues A, Tostões RM, Leite S, Zimmerman H, Carrondo MJT, et al. Extending hepatocyte functionality for drug-testing applications using high-viscosity alginate-encapsulated three-dimensional cultures in bioreactors. *Tissue Eng Part C Methods*. (2010) 16:1223–32. doi: 10.1089/ten.tec.2009.0784

9. Nino-Vasquez IA, Muñiz-Márquez D, Ascacio-Valdés JA, Contreras-Esquivel JC, Aguilar CN, Rodríguez-Herrera R, et al. Co-microencapsulation: a promising multi-approach technique for enhancement of functional properties. *Bioengineered*. (2022) 13:5168–89. doi: 10.1080/21655979.2022.2037363

10. Tran NM, Dufresne M, Helle F, Hoffmann TW, François C, Brochot E, et al. Alginate hydrogel protects encapsulated hepatic HuH-7 cells against hepatitis C virus and other viral infections. *PloS One*. (2014) 9:e109969. doi: 10.1371/journal.pone.0109969

11. Lan SF, Safiejko-Mroczka B, Starly B. Long-term cultivation of HepG2 liver cells encapsulated in alginate hydrogels: a study of cell viability, morphology and drug metabolism. *Toxicol In Vitro*. (2010) 24:1314–23. doi: 10.1016/j.tiv.2010.02.015

12. Azandeh S, Nejad DB, Bayati V, Shakoor F, Varaa N, Cheraghian B. High mannoronic acid containing alginate affects the differentiation of Wharton's jelly-derived stem cells to hepatocyte-like cell. *J Adv Pharm Technol Res*. (2019) 10:9–15. doi: 10.4103/japtr.JAPTR_312_18

13. Jitraruch S, Dhawan A, Hughes RD, Filippi C, Soong D, Philippeos C, et al. Alginate microencapsulated hepatocytes optimised for transplantation in acute liver failure. *PloS One*. (2014) 9:e113609. doi: 10.1371/journal.pone.0113609

14. Pasqua M, Pereira U, Messina A, Lartigue C, Vigneron P, Dubart-Kupperschmitt A, et al. HepaRG self-assembled spheroids in alginate beads meet the clinical needs for bioartificial liver. *Tissue Eng Part A*. (2020) 26:613–22. doi: 10.1089/ten.tea.2019.0262

15. Durkut S, Elcin AE, Elcin YM. In vitro evaluation of encapsulated primary rat hepatocytes pre- and post-cryopreservation at -80 degrees C and in liquid nitrogen. *Artif Cells Nanomed Biotechnol*. (2015) 43:50–61. doi: 10.3109/21691401.2013.837476

16. Capone SH, Dufresne M, Rechel M, Fleury M-J, Salsac A-V, Paullier P, et al. Impact of alginate composition: from bead mechanical properties to encapsulated HepG2/C3A cell activities for in vivo implantation. *PloS One*. (2013) 8:e62032. doi: 10.1371/journal.pone.0062032

17. Nayak S, Dey S, Kundu SC. Silk sericin-alginate-chitosan microcapsules: hepatocytes encapsulation for enhanced cellular functions. *Int J Biol Macromol*. (2014) 65:258–66. doi: 10.1016/j.ijbiomac.2014.01.042

18. Tian M, Han B, Tan H, You C. Preparation and characterization of galactosylated alginate-chitosan oligomer microcapsule for hepatocytes microencapsulation. *Carbohydr Polym*. (2014) 112:502–11. doi: 10.1016/j.carbpol.2014.06.025

19. He Y, Liu C, Xia X, Liu L. Conformal microcapsules encapsulating microcarrier-L02 cell complexes for treatment of acetaminophen-induced liver injury in rats. *J Mater Chem B*. (2017) 5:1962–70. doi: 10.1039/C6TB03033E

20. Siltanen C, Diakatou M, Lowen J, Haque A, Rahimian A, Stybayeva G, et al. One step fabrication of hydrogel microcapsules with hollow core for assembly and cultivation of hepatocyte spheroids. *Acta Biomater*. (2017) 50:428–36. doi: 10.1016/j.actbio.2017.01.010

21. Khodabakhsh Aghdam S, Khoshfetrat AB, Rahbarghazi R, Jafarizadeh-Malmiri H, Khaksar M. Collagen modulates functional activity of hepatic cells inside alginate-galactosylated chitosan hydrogel microcapsules. *Int J Biol Macromol*. (2020) 156:1270–8. doi: 10.1016/j.ijbiomac.2019.11.164

22. Zhang Y, Lu J, Li Z, Zhu D, Yu X, Li L. Enhanced cellular functions of hepatocytes in the hyaluronate-alginate-chitosan microcapsules. *Int J Artif Organs*. (2021) 44:340–9. doi: 10.1177/0391398820959345

23. Yamada K, Aoki T, Enami Y, Tashiro Y, Zehaou Z, Koizumi T, et al. An improved encapsulation method for cryopreserving hepatocytes for functional transplantation using a thermo-reversible gelation polymer. *In Vivo*. (2020) 34:2309–16. doi: 10.21873/invivo.12043

24. Lu J, Zhang X, Li J, Yu L, Chen E, Zhu D. A new fluidized bed bioreactor based on diversion-type microcapsule suspension for bioartificial liver systems. *PloS One*. (2016) 11:e0147376. doi: 10.1371/journal.pone.0147376

25. Shi XL, Zhang Y, Gu J-Y, Ding Y-T. Coencapsulation of hepatocytes with bone marrow mesenchymal stem cells improves hepatocyte-specific functions. *Transplantation*. (2009) 88:1178–85. doi: 10.1097/TP.0b013e3181bc288b

26. Fitzpatrick E, Wu Y, Dhadda P, Hughes RD, Mitry RR, Qin H, et al. Coculture with mesenchymal stem cells results in improved viability and function of human hepatocytes. *Cell Transplant*. (2015) 24:73–83. doi: 10.3727/096368913X674080

27. Li CY, Stevens KR, Schwartz RE, Alejandro BS, Huang JH, Bhatia SN. Micropatterned cell-cell interactions enable functional encapsulation of primary hepatocytes in hydrogel microtissues. *Tissue Eng Part A*. (2014) 20:2200–12. doi: 10.1089/ten.tea.2013.0667

28. Elisa M, Pimenta J, Szabó L, Noverraz F, Passemard S, Meier RPH, et al. Beneficial effects of human mesenchymal stromal cells on porcine hepatocyte viability and albumin secretion. *J Immunol Res*. (2018) 2018:1078547. doi: 10.1155/2018/1078547

29. Chen Y, Yu C, Lv G, Cao H, Yang S, Zhang Y, et al. Rapid large-scale culturing of microencapsulated hepatocytes: a promising approach for cell-based hepatic support. *Transplant Proc*. (2014) 46:1649–57. doi: 10.1016/j.transproceed.2014.03.002

30. Zheng MH, Lin H-L, Qiu L-X, Cui Y-L, Sun Q-F, Chen Y-P. Mixed microencapsulation of rat primary hepatocytes and Sertoli cells improves the metabolic function in a D-galactosamine and lipopolysaccharide-induced rat model of acute liver failure. *Cytotherapy*. (2009) 11:326–9. doi: 10.1080/14653240802582091

31. Satta S, Shahabipour F, Gao W, Lentz SR, Perlman S, Ashammakhi N, et al. Engineering viral genomics and nano-liposomes in microfluidic platforms for patient-specific analysis of SARS-CoV-2 variants. *Theranostics*. (2022) 12:4779–90. doi: 10.7150/thno.72339

32. Gao Y, Xu J, Sun B, Jiang H-C. Microencapsulated hepatocytes and islets as in vivo bioartificial liver support system. *World J Gastroenterol*. (2004) 10:2067–71. doi: 10.3748/wjg.v10.i14.2067

33. Kong D. Co-encapsulation of HNF4α overexpressing UMSCs and human primary hepatocytes ameliorates mouse acute liver failure. *Stem Cell Res Ther*. (2020) 11(1):449. doi: 10.1186/s13287-020-01962-7

34. Liu ZC, Chang TI. Biotechnology. Increased viability of transplanted hepatocytes when hepatocytes are co-encapsulated with bone marrow stem cells using a novel method. *Artif Cells Blood Substit Immobil Biotechnol*. (2002) 30(2):99–112. doi: 10.1081/BIO-120003191

35. Nishikawa M, Kojima N, Komori K, Yamamoto T, Fujii T, Sakai Y. Enhanced maintenance and functions of rat hepatocytes induced by combination of on-site oxygenation and coculture with fibroblasts. *J Biotechnol*. (2008) 133:253–60. doi: 10.1016/j.jbiotec.2007.08.041

36. Liu ZC, Chang TMS. Coencapsulation of hepatocytes and bone marrow stem cells: in vitro conversion of ammonia and in vivo lowering of bilirubin in hyperbilirubemia Gunn rats. *Int J Artif Organs*. (2003) 26(6):491–7. doi: 10.1177/039139880302600607

37. Isoda K, Kojima M, Takeda M, Higashiyama S, Kawase M, Yagi K. Maintenance of hepatocyte functions by coculture with bone marrow stromal cells. *J Biosci Bioengineering*. (2004) 97:343–6. doi: 10.1263/jbb.97.343

38. Goh Takayama AT, Okano T. Identification of differentially expressed genes in hepatocyte/endothelial cell co-culture system. *J Tissue Eng*. (2007) 13:159. doi: 10.1089/ten.2007.13.159

39. Valeria I, Dhawan A, Masmoudi F, Lee CA, Fernandez-Dacosta R, Walker S, et al. A new high throughput screening platform for cell encapsulation in alginate hydrogel shows improved hepatocyte functions by mesenchymal stromal cells co-encapsulation. *Front Med*. (2018) 5:216. doi: 10.3389/fmed.2018.00216

40. Ding DC, Shyu WC, Lin SZ. Mesenchymal stem cells. *Cell Transplant*. (2011) 20:5–14. doi: 10.3727/096368910X

41. Kim K, Ohashi K, Utoh R, Kano K, Okano T. Preserved liver-specific functions of hepatocytes in 3D co-culture with endothelial cell sheets. *Biomaterials*. (2012) 33:1406–13. doi: 10.1016/j.biomaterials.2011.10.084

42. Chan HF, Zhang Y, Leong KW. Efficient one-step production of microencapsulated hepatocyte spheroids with enhanced functions. *Small*. (2016) 12:2720–30. doi: 10.1002/smll.201502932

43. Lee HJ, Son MJ, Ahn J, Oh SJ, Lee M, Kim A, et al. Elasticity-based development of functionally enhanced multicellular 3D liver encapsulated in hybrid hydrogel. *Acta Biomater*. (2017) 64:67–79. doi: 10.1016/j.actbio.2017.09.041

44. Cui J, Wang H, Shi Q, Sun T, Huang Q, Fukuda T. Multicellular co-culture in three-dimensional gelatin methacryloyl hydrogels for liver tissue engineering. *Molecules*. (2019) 24(9):1762. doi: 10.3390/molecules24091762

45. Chang SH, Huang HH, Kang PL, Wu YC, Chang M-H, Kuo Ming S. In vitro and in vivo study of the application of volvox spheres to co-culture vehicles in liver tissue engineering. *Acta Biomater*. (2017) 63:261–73. doi: 10.1016/j.actbio.2017.09.028

46. Kim M, Lee JY, Jones CN, Revzin A, Tae G. Heparin-based hydrogel as a matrix for encapsulation and cultivation of primary hepatocytes. *Biomaterials*. (2010) 31:3596–603. doi: 10.1016/j.biomaterials.2010.01.068

47. Gevaert E, Billiet T, Declercq H, Dubruel P, Cornelissen R. Galactose-functionalized gelatin hydrogels improve the functionality of encapsulated HepG2 cells. *Macromol Biosci*. (2014) 14:419–27. doi: 10.1002/mabi.201300320

48. Lee HJ, Ahn J, Jung C-R, Jeung Y-J, Cho H-S, Son MJ, et al. Optimization of 3D hydrogel microenvironment for enhanced hepatic functionality of primary human hepatocytes. *Biotechnol Bioeng*. (2020) 117:1864–76. doi: 10.1002/bit.27328

49. Wang Y, Liu H, Zhang M, Wang H, Chen W, Qin J. One-step synthesis of composite hydrogel capsules to support liver organoid generation from hiPSCs. *Biomater Sci*. (2020) 8:5476–88. doi: 10.1039/D0BM01085E

50. Tirella A, Marca La M, Brace L-A, Mattei G, Aylott JW, Ahluwalia A, et al. Nano-in-micro self-reporting hydrogel constructs. *J BioMed Nanotechnol*. (2015) 11:1451–60. doi: 10.1166/jbn.2015.2085

51. Khanal S, Bhattarai SR, Sankar J, Bhandari RK, Macdonald JM, Bhattarai N. Nano-fibre integrated microcapsules: A nano-in-micro platform for 3D cell culture. *Sci Rep*. (2019) 9:13951. doi: 10.1038/s41598-019-50380-0

52. Zheng Z, Wang H, Li J, Shi Q, Cui J, Sun T, et al. 3D construction of shape-controllable tissues through self-bonding of multicellular microcapsules. *ACS Appl Mater Interfaces*. (2019) 11:22950–61. doi: 10.1021/acsami.9b05108

53. Yu W, Zhang W, Chen Y, Song X, Tong W, Mao Z, et al. Cellular uptake of poly (allylamine hydrochloride) microcapsules with different deformability and its influence on cell functions. *J Colloid Interface Sci*. (2016) 465:149–57. doi: 10.1016/j.jcis.2015.11.065

54. Cui J, Wang H, Zheng Z, Shi Q, Sun T, Huang Q, et al. Fabrication of perfusable 3D hepatic lobule-like constructs through assembly of multiple cell type laden hydrogel microstructures. *Biofabrication*. (2018) 11:015016. doi: 10.1088/1758-5090/aaf3c9

55. Liu Z, Takeuchi M, Nakajima M, Hu C, Hasegawa Y, Huang Q, et al. Three-dimensional hepatic lobule-like tissue constructs using cell-microcapsule technology. *Acta Biomater*. (2017) 50:178–87. doi: 10.1016/j.actbio.2016.12.020

56. Moriyama K, Naito S, Wakabayashi R, Goto M, Kamiya N. Enzymatically prepared redox-responsive hydrogels as potent matrices for hepatocellular carcinoma cell spheroid formation. *Biotechnol J*. (2016) 11:1452–60. doi: 10.1002/biot.201600087

57. Agarwal T, Narayan R, Maji S, Ghosh SK, Maiti TK. Decellularized caprine liver extracellular matrix as a 2D substrate coating and 3D hydrogel platform for vascularized liver tissue engineering. *J Tissue Eng Regener Med*. (2018) 12:e1678–90. doi: 10.1002/term.2594

58. Zhang X, Lu J, He B, Tang L, Liu X, Zhu D, et al. A tryptophan derivative, ITE, enhances liver cell metabolic functions in vitro. *Int J Mol Med*. (2017) 39:101–12. doi: 10.3892/ijmm.2016.2825

59. Ravichandran A, Murekatete B, Moedder D, Meinert C, Bray LJ. Photocrosslinkable liver extracellular matrix hydrogels for the generation of 3D liver microenvironment models. *Sci Rep*. (2021) 11:15566. doi: 10.1038/s41598-021-94990-z

60. Zhang J, Hu X, Zheng G, Yao H, Liang H. In vitro and in vivo antitumor effects of lupeol-loaded galactosylated liposomes. *Drug Delivery*. (2021) 28:709–18. doi: 10.1080/10717544.2021.1905749

61. Leroux G, Neumann M, Meunier CF, Fattaccioli A, Michiels C, Arnould T, et al. Hybrid alginate@TiO2 porous microcapsules as a reservoir of animal cells for cell therapy. *ACS Appl Mater Interfaces*. (2018) 10:37865–77. doi: 10.1021/acsami.8b15483

62. Sk MM, Das P, Panwar A, Tan LP. Synthesis and characterization of site selective photo-crosslinkable glycidyl methacrylate functionalized gelatin-based 3D hydrogel scaffold for liver tissue engineering. *Mater Sci Eng C Mater Biol Appl*. (2021) 123:111694. doi: 10.1016/j.msec.2020.111694

63. Tostoes RM, Leite SB, Miranda JP, Sousa M, Wang DIC, Carrondo MJT, et al. Perfusion of 3D encapsulated hepatocytes–a synergistic effect enhancing long-term functionality in bioreactors. *Biotechnol Bioeng*. (2011) 108:41–9. doi: 10.1002/bit.22920

64. Rebelo SP, Costa R, Estrada M, Shevchenko V, Brito C, Alves PM. HepaRG microencapsulated spheroids in DMSO-free culture: novel culturing approaches for enhanced xenobiotic and biosynthetic metabolism. *Arch Toxicol*. (2015) 89:1347–58. doi: 10.1007/s00204-014-1320-9

65. Maguire T, Novik E, Schloss R, Yarmush M. Alginate-PLL microencapsulation: effect on the differentiation of embryonic stem cells into hepatocytes. *Biotechnol Bioeng*. (2006) 93:581–91. doi: 10.1002/bit.20748

66. MacPherson D, Bram Y, Park J, Schwartz RE. Peptide-based scaffolds for the culture and maintenance of primary human hepatocytes. *Sci Rep*. (2021) 11:6772. doi: 10.1038/s41598-021-86016-5

67. Chen L, Zhang Y, Li S, Wang X, Li N, Wang Y. Effect of plasma components on the stability and permeability of microcapsule. *J BioMed Mater Res A*. (2014) 102:2408–16. doi: 10.1002/jbm.a.34907

68. Liu T, Yi S, Liu G, Hao X, Du T, Chen J, et al. Aqueous two-phase emulsions-templated tailorable porous alginate beads for 3D cell culture. *Carbohydr Polym*. (2021) 258:117702. doi: 10.1016/j.carbpol.2021.117702

69. Nagata S, Ozawa F, Nie M, Takeuchi S. 3D culture of functional human iPSC-derived hepatocytes using a core-shell microfiber. *PloS One*. (2020) 15:e0234441. doi: 10.1371/journal.pone.0234441

70. Mai G, Nguyen TH, Morel P, Mei J, Andres A, Bosco D, et al. Treatment of fulminant liver failure by transplantation of microencapsulated primary or immortalized xenogeneic hepatocytes. *Xenotransplantation*. (2005) 12:457–64. doi: 10.1111/j.1399-3089.2005.00248.x

71. Hang H, Shi X, Gu G, Wu Y, Gu J, Ding Y. In vitro analysis of cryopreserved alginate-poly-L-lysine-alginate-microencapsulated human hepatocytes. *Liver Int*. (2010) 30:611–22. doi: 10.1111/liv.2010.30.issue-4

72. Kilbride P, Mahbubani KT, Saeb-Parsy K, Morris GJ. Engaging cold to upregulate cell proliferation in alginate-encapsulated liver spheroids. *Tissue Eng Part C Methods*. (2017) 23:455–64. doi: 10.1089/ten.tec.2017.0131

73. Koizumi T, Aoki T, Kobayashi Y, Yasuda D, Izumida Y, Jin Z, et al. Long-term maintenance of the drug transport activity in cryopreservation of microencapsulated rat hepatocytes. *Cell Transplant*. (2007) 16:67–73. doi: 10.3727/000000007783464489

74. Jitraruch S, Dhawan A, Hughes RD, Filippi C, Lehec SC, Glover L, et al. Cryopreservation of hepatocyte microbeads for clinical transplantation. *Cell Transplant*. (2017) 26:1341–54. doi: 10.1177/0963689717720050

75. Lee JH, Lee DH, Park JK, Kim SK, Kwon CHD, Lee SK. Effect of fulminant hepatic failure porcine plasma supplemented with essential components on encapsulated rat hepatocyte spheroids. *Transplant Proc*. (2012) 44:1009–11. doi: 10.1016/j.transproceed.2012.01.106

76. Rahman TM, Diakanov I, Selden C, Hodgson H. Co-transplantation of encapsulated HepG2 and rat Sertoli cells improves outcome in a thioacetamide induced rat model of acute hepatic failure. *Transpl Int*. (2010) 18(8):1001–9. doi: 10.1111/j.1432-2277.2005.00156.x

77. Yang G, Mahadik B, Mollot T, Pinsky J, Jones A, Robinson A, et al. Engineered liver tissue culture in an in vitro tubular perfusion system. *Tissue Eng Part A*. (2020) 26:1369–77. doi: 10.1089/ten.tea.2020.0213

78. Kukla DA, Crampton AL, Wood DK, Khetani SR. Microscale collagen and fibroblast interactions enhance primary human hepatocyte functions in three-dimensional models. *Gene Expr*. (2020) 20:1–18. doi: 10.3727/105221620X15868728381608

79. Zhang Y, Chen XM, Sun DL. Effects of coencapsulation of hepatocytes with adipose-derived stem cells in the treatment of rats with acute-on-chronic liver failure. *Int J Artif Organs*. (2014) 37:133–41. doi: 10.5301/ijao.5000284

80. Teng Y, Wang Y, Li S, Wang W, Gu R, Guo X, et al. Treatment of acute hepatic failure in mice by transplantation of mixed microencapsulation of rat hepatocytes and transgenic human fetal liver stromal cells. *Tissue Eng Part C Methods*. (2010) 16 (5):1125–34. doi: 10.1089/ten.tec.2009.0374

81. Liyuan, Wang J, Wen X, Wang H, Wang Y, Lin Q, et al. Transplantation of co-microencapsulated hepatocytes and HUVECs for treatment of fulminant hepatic failure. *Int J Artif Organs*. (2012) 35(6):458–65. doi: 10.5301/ijao.5000092

82. Liu WM, Zhou X, Chen C-Y, Lv D-D, Huang W-J, Peng Y, et al. Establishment of functional liver spheroids from human hepatocyte-derived liver progenitor-like cells for cell therapy. *Front Bioeng Biotechnol*. (2021) 9:738081. doi: 10.3389/fbioe.2021.738081

83. Song W, Lu Y-C, Frankel AS, An D, Schwartz RE, Ma M. Engraftment of human induced pluripotent stem cell-derived hepatocytes in immunocompetent mice via 3D co-aggregation and encapsulation. *Sci Rep*. (2015) 5:16884. doi: 10.1038/srep16884

84. Yuan X, Wu J, Sun Z, Cen J, Shu Y, Wang C, et al. Preclinical efficacy and safety of encapsulated proliferating human hepatocyte organoids in treating liver failure. *Cell Stem Cell*. (2024) 31(4):484–98.e5. doi: 10.1016/j.stem.2024.02.005

85. Mei J, Mai G, Baertschiger R, Gonelle-Gispert C, Serre-Beinier V, et al. Improved survival of fulminant liver failure by transplantation of microencapsulated cryopreserved porcine hepatocytes in mice. *Cell Transplant*. (2009) 18:101–10. doi: 10.3727/096368909788237168

86. Sgroi A, Mai G, Morel P, Baertschiger RM, Gonelle-Gispert C, Serre-Beinier V, et al. Transplantation of Encapsulated Hepatocytes during Acute Liver Failure Improves Survival without Stimulating Native Liver Regeneration. *Cell Transplant*. (2011) 20:1791–803. doi: 10.3727/096368911X564976

87. Meier RPH, Navarro-Alvarez N, Morel P, Schuurman H-J, Strom S, Bühler LH. Current status of hepatocyte xenotransplantation. *Int J Surg*. (2015) 23:273–9. doi: 10.1016/j.ijsu.2015.08.077

88. Ham DS, Song M-S, Park H-S, Rhee M, Yang HK, Lee S-H, et al. Successful xenotransplantation with re-aggregated and encapsulated neonatal pig liver cells for treatment of mice with acute liver failure. *Xenotransplantation*. (2015) 22:249–59. doi: 10.1111/xen.12177

89. Machaidze Z, Yeh H, Wei L, Schuetz C, Carvello M, Sgroi A, et al. Testing of microencapsulated porcine hepatocytes in a new model of fulminant liver failure in baboons. *Xenotransplantation*. (2017) 24(3). doi: 10.1111/xen.12297

90. Varaa N, Azandeh S, Khorsandi L, Nejad DB, Bayati V, Bahreini A. Ameliorating effect of encapsulated hepatocyte-like cells derived from umbilical cord in high mannuronic alginate scaffolds on acute liver failure in rats. *Iran J Basic Med Sci*. (2018) 21:928–35. doi: 10.22038/IJBMS.2018.27928.6847

91. Bonavita AG, Quaresma K, Cotta-de-Almeida V, Pinto MA, Saraiva RM, Alves LA. Hepatocyte xenotransplantation for treating liver disease. *Xenotransplantation*. (2010) 17:181–7. doi: 10.1111/xen.2010.17.issue-3

92. Gu Y, Zheng X, Ji J. Liver cancer stem cells as a hierarchical society: yes or no? *Acta Biochim Biophys Sin (Shanghai)*. (2020) 52:723–35. doi: 10.1093/abbs/gmaa050

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 03 June 2024
DOI 10.3389/fimmu.2024.1391504

OPEN ACCESS

EDITED BY
Lisha Mou,
Shenzhen Second People's Hospital, China

REVIEWED BY
Pradeep Shrestha,
University of Texas MD Anderson Cancer Center, United States
Ashwin Ajith,
Augusta University, United States

*CORRESPONDENCE
Yi Sun
sunyimao123@hotmail.com
Xinli Mao
maoxl@emzemed.com
Shao-wei Li
li_shaowei81@hotmail.com

†These authors have contributed equally to this work

RECEIVED 26 February 2024
ACCEPTED 20 May 2024
PUBLISHED 03 June 2024

CITATION
Wang Q, Huang Y-x, Liu L, Zhao X-h, Sun Y, Mao X and Li S-w (2024) Pancreatic islet transplantation: current advances and challenges.
Front. Immunol. 15:1391504.
doi: 10.3389/fimmu.2024.1391504

Pancreatic islet transplantation: current advances and challenges

Qi Wang[1†], Yu-xi Huang[1†], Long Liu[2†], Xiao-hong Zhao[3], Yi Sun[4*], Xinli Mao[5,6*] and Shao-wei Li[5,6*]

[1]Department of Hepatobiliary and Pancreatic Surgery, Taizhou Hospital of Zhejiang Province Affiliated to Wenzhou Medical University, Linhai, Zhejiang, China, [2]Department of Hepatobiliary and Pancreatic Surgery, Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China, [3]Department of Pharmacy, Taizhou Hospital, Zhejiang University, Taizhou, Zhejiang, China, [4]MRL Global Medical Affairs, MSD China, Shanghai, China, [5]Department of Gastroenterology, Taizhou Hospital of Zhejiang Province Affiliated to Wenzhou Medical University, Linhai, Zhejiang, China, [6]Key Laboratory of Minimally Invasive Techniques and Rapid Rehabilitation of Digestive System Tumor of Zhejiang Province, Taizhou Hospital Affiliated to Wenzhou Medical University, Linhai, Zhejiang, China

Diabetes is a prevalent chronic disease that traditionally requires severe reliance on medication for treatment. Oral medication and exogenous insulin can only temporarily maintain blood glucose levels and do not cure the disease. Most patients need life-long injections of exogenous insulin. In recent years, advances in islet transplantation have significantly advanced the treatment of diabetes, allowing patients to discontinue exogenous insulin and avoid complications.Long-term follow-up results from recent reports on islet transplantation suggest that they provide significant therapeutic benefit although patients still require immunotherapy, suggesting the importance of future transplantation strategies. Although organ shortage remains the primary obstacle for the development of islet transplantation, new sources of islet cells, such as stem cells and porcine islet cells, have been proposed, and are gradually being incorporated into clinical research. Further research on new transplantation sites, such as the subcutaneous space and mesenteric fat, may eventually replace the traditional portal vein intra-islet cell infusion. Additionally, the immunological rejection reaction in islet transplantation will be resolved through the combined application of immunosuppressant agents, islet encapsulation technology, and the most promising mesenchymal stem cells/regulatory T cell and islet cell combined transplantation cell therapy. This review summarizes the progress achieved in islet transplantation, and discusses the research progress and potential solutions to the challenges faced.

KEYWORDS

pancreatic islet, transplantation, long-term outcomes, MSC/Treg, co-transplantation

1 Introduction

Type 1 diabetes (T1D) is a chronic progressive metabolic disorder characterized by hyperglycemia due to destruction of pancreatic β-cells leading to severe insulin deficiency (1). In the early stages, blood sugar levels can be controlled within the normal range using oral hypoglycemic drugs or insulin injections. However, for some patients with advanced diabetes, these interventions are limited in effectiveness and cannot prevent complications, such as metabolic disorders, vascular diseases, and nerve damage. Severe cases can lead to limb necrosis, blindness, kidney failure, and life-threatening conditions (2–4). Although significant progress has been made in diabetes treatment in recent years with new technologies and medications, such as insulin pumps and continuous glucose monitoring devices, the treatment of diabetes remains a significant burden for patients because of the need for dynamic blood sugar monitoring and adjustment. Therefore, searching for new treatment methods is a major issue in the field of diabetes.

Pancreatic islet transplantation (IT) is a procedure that involves the purification of pancreatic islet cells from a donor pancreas, whether it is xenogeneic and their infusion into the patient's body, mainly through the portal vein. This establishes an endogenous glucose-dependent insulin secretion system, restoring physiological insulin secretion patterns and achieving real-time, accurate blood glucose control. In the long term, it can improve diabetic complications and enable insulin independence, ultimately aiming to cure diabetes. It is considered an ideal solution for diabetes (5). IT has garnered widespread attention as an effective treatment for diabetes. However, many difficulties and challenges have hindered its development (6).

Organ shortage is a global issue and hampers the development of pancreatic IT. Approximately 8,000 organ donations occur annually, but less than one-third of the pancreatic organs are usable for IT (7, 8). The long-term clinical prognosis of patients undergoing traditional portal vein transplantation is poor. Studies have shown that post-transplantation inflammatory and immune rejection reactions can lead to up to 60% pancreatic islet dysfunction or necrosis. Furthermore, complications such as portal hypertension, bleeding, and thrombosis can occur during the portal vein transplantation procedure (9).

In response to these issues, numerous researchers have proposed solutions, and the main research directions to address the shortage of pancreatic islet organs focus on stem cell-derived and porcine-derived islet cells. In terms of selecting new transplant sites, options such as a subcutaneous pocket and the greater omentum have certain advantages compared to the traditional portal vein injection method. In addition, islet encapsulation technology and cellular therapy for combined transplantation of MSC/Treg and islet cells are also under active development to induce immune tolerance in transplant recipients.

We herein report an overview of the current long-term prognosis of patients following IT. Then, we discuss and elaborate on the challenges faced in the IT process and the recent progress of the corresponding solutions. We hope that this information will offer guidance and reference for further research in the field of IT.

2 Current outcomes of pancreatic islet transplantation

Clinical IT has been carried out since the 1970s (10), however, for various reasons, its clinical efficacy has not been satisfactory. It was not until 2000 that Shapiro et al. (11) proposed and established a set of standards, including donor selection, transplantation of islet equivalents, and postoperative immunosuppressive regimens. They used a large number of isolated islet cells for transplantation and implemented a new protocol after surgery using a corticosteroid-free regimen and reduced doses of calcium channel blockers (sirolimus, low-dose tacrolimus, and daclizumab), known as the "Edmonton protocol" (11). Once this protocol was promoted, clinical results showed significant improvement, marking an important milestone in clinical IT. In 2006, a clinical islet transplantation trial using the Edmonton protocol (NCT00014911) was published, in which 36 subjects with T1D were enrolled at nine transplant centers for islet transplantation using the Edmonton protocol, with insulin independence and good glycemic control as the endpoint 1 year after transplantation. Results showed that a total of 16 subjects met the primary endpoint, including 5 subjects who remained insulin independent 2 years after transplantation (12). This clinical trial suggests that islet transplantation using the Edmonton protocol can restore long-term endogenous insulin production and stabilize blood glucose levels in T1D patients, but insulin independence may not persist. It may be necessary to continue improving the immunosuppressive regimen to achieve longer insulin independence after islet transplantation. We summarize some clinical trials of immunosuppressive regimen (Table 1) and using porcine islets in non-human primates (Table 2).

In recent years, several research teams have published studies on the long-term progress of IT, affirming its therapeutic effects and providing new ideas for future treatment protocols (Figure 1). In 2016, Bernhard et al. published a phase III clinical trial for the treatment of severe hypoglycemic complications in T1DM patients through IT. The trial was conducted at 8 centers in North America and included 48 T1DM patients who had been suffering for over 5 years. During the trial, each patient underwent one or more ITs. The primary endpoints of the trial were achieving HbA1c <7.0% (53 mmol/mol) within the first year after the first transplant and avoiding severe hypoglycemic events (SHEs) from day 28 to day 365. The results showed that 87.5% of the participants successfully reached the primary endpoints within one year. IT enables blood sugar control for patients with refractory SHEs and should be considered when other treatments are ineffective (33). In 2023, the team conducted a follow-up investigation of 398 patients with T1DM and SHEs registered in the Collaborative Islet Transplant Registry (CITR). They identified 4 factors that are most beneficial for IT: patients ≥35 years old, infusion of 325,000 islet equivalents, immunosuppression with T cell depletion or TNF-α inhibition, and the use of rapamycin (mTOR) and calcineurin inhibitors. When islet transplant recipients reach the milestone of 5 years after their last islet cell infusion, approximately 95% of patients who meet

TABLE 1 Different immunosuppressive regimens in islet transplantation.

Immunosuppression therapy	Result	References
Sirolimus, tacrolimus, and daclizumab	Achieved sustained insulin independence for 11.9 months	(11, 13)
Sirolimus or mycophenolate, belatacept (BELA) or efalizumab (EFA)	Achieving insulin independence after one or two islet transplants	(14)
Thymoglobulin and sirolimus, efalizumab, mycophenolic acid (MMF)	All patients achieved insulin independence and complete remission of hypoglycemic episodes after the last islet transplant	(15)
Anti-CD3 mAb and sirolimus, maintained with sirolimus and reduced-dose tacrolimus	Four of six recipients achieved and maintained insulin independence with an increased percentage of CD4+ T cells	(16)
Antithymocyte globulin (ATG), daclizumab, and etanercept, maintained with mycophenolate mofetil, sirolimus, and no or low-dose tacrolimus	Insulin independence and absence of hypoglycemia was achieved in all 8 recipients	(17)
Daclizumab, sirolimus, tacrolimus, etanercept, exenatide	Improves islet graft function and contributes to insulin independence with reduced islets	(18)
Thymoglobulin induction, and doubleblockage of IL-1β and TNF-α as well as sirolimus-free immunosuppression	Only one islet infusion is required, significantly improving the efficacy of clinical islet transplantation	(19)
Rapamycin, ATG, steroids and interleukin-1Ra, rapamycin, mycophenolate mofetil treatment as maintenance therapy	This regimen is feasible and safe but less efficient in maintaining graft survival than other regimens based on T-cell depletion	(20)
Induction immunosuppression with T cell depletion and/or TNF-α inhibition; and maintenance with both mechanistic target of rapamycin (mTOR) and calcineurin inhibitors	Safe to use and exerts a great and significant benefit in blood glucose control	(21)
Alemtuzumab, basiliximab, maintained withtacrolimus, mycophenolatemofetil, and prednisolone	This protocol for postrenal islet transplantation significantly improves islet allograft function and improves glycemic control	(22)

these 4 common factors experience no SHEs and greatly benefit from improved glycemic control (13).

In 2022, Marfil-Garza et al. from the University of Alberta, Edmonton, Canada, published a study on the long-term results of pancreatic islet cell transplantation over a period of 20 years. This is the largest cohort study to date on the long-term outcomes of IT, including 255 patients from the Edmonton Protocol. This study showed that despite the need for chronic immunosuppression therapy, islet cell transplantation demonstrated good long-term safety. In this study, the median follow-up time was 7.4 years, with 90% patient survival and a median graft survival of 5.9 years. Patients surviving post-transplant exhibit better insulin sensitivity and more stable blood glucose control than non-survivors (34). This study is significant for understanding the long-term effects of islet cell transplantation and for identifying predictive factors. However, further research is needed to validate these results and to continue to evaluate the risks and benefits of IT for better treatment choices for patients.

TABLE 2 Immunosuppressive protocol for transplantation of porcine pancreatic islets into nonhuman primates.

Immunosuppressive drugs	Graft survival time	References
Anti-CD154 mAb, basiliximab, belatacept, sirolimus	>140 days	(23)
CD154-specific and CD25-specific mAb, FTY720 (or tacrolimus), everolimus and leflunomide	>100 days	(24)
CD40-specific monoclonal antibody (Chi220), basiliximab, belatacept, sirolimus	203 days	(25)
Belatacept and mycophenolate, LFA-1 blockade, basiliximab, tacrolimus,	111 days	(26)
Cobra venom factor (CVF), anti-CD154 mAb, low-dose Sirolimus, anti-thymocyte globulin (ATG),Tregs	603 days	(27)
ATG, anti-CD40 mAb, CVF, adalimumab, sirolimus, with or without belatacept or tacrolimus	60 days	(28)

In a retrospective, multicenter, observational cohort study, 1210 patients from the Pancreatic Islet Transplantation Collaborative Registry at 39 centers worldwide were included. The study demonstrated a linear inverse relationship between primary graft function (PGF) at one month post-most recent IT and the five-year cumulative incidence of adverse outcomes. This suggests an association between early transplantation potential and long-term clinical significance, which has important implications for β-cell replacement therapies. Anticipated clinical outcomes can guide personalized decisions regarding repeat islet injections based on a predefined islet quality threshold, informing current practice. In future trials, PGF may serve as an early and reliable surrogate endpoint for successful IT. These findings highlight the potential of evaluating and optimizing early IT to improve current β-cell replacement outcomes through an enhanced islet survival and function post-transplant. This can enhance the effectiveness of IT and improve patient prognoses (35).

In conclusion, the latest research and clinical data unequivocally support the safety and efficacy of pancreatic islet cell transplantation in T1DM treatment. Furthermore, these studies offer promising new directions for further optimization of IT and for achieving long-term success.

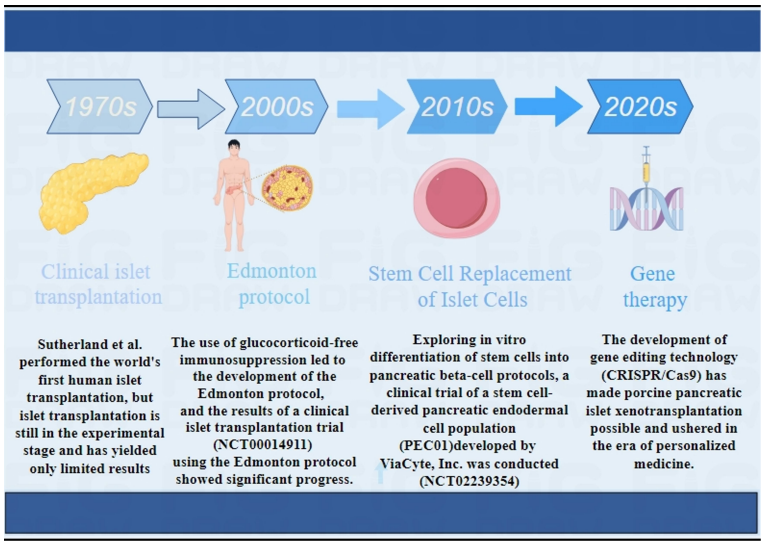


FIGURE 1
Main development process of clinical islet transplantation.1970S (10), 2000S (11, 12), 2010S (29, 30), 2020S (31, 32).

3 β-cell replacement options: stem cells and porcine islets

Pancreatic IT holds great promise in the treatment of T1DM. However, the scarcity of pancreatic islets limits the development of this technique. Several research teams have proposed different solutions. Currently, the main focus of pancreatic cell replacement strategies is on stem cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), as well as porcine islets.

3.1 ESCs/iPSCs differentiate into islet β-cells

The strategy for *in vitro* differentiation of ESCs/iPSCs into pancreatic cells mimics the molecular regulatory mechanisms of pancreatic development *in vivo*. It involves the use of a combination of growth factors and small molecules to activate developmental signaling pathways and transcription factor networks. Through staged induction, pancreatic progenitor cells and endocrine progenitor cells eventually differentiate into mature endocrine cells (α, β, δ) cells (36–39). D'Amour et al. first attempted to establish a protocol for generating hormone-expressing cells that can synthesize and release multiple hormones (40). Rezania et al. reported a seven-step differentiation protocol, in which the resulting cells expressed key markers of mature β-cells, such as MAFA, PDX1, NKX6.1, and INS, and exhibited similar functionality to human islets (Figure 2) (41). Subsequently, Pagliuca et al. utilized human ESCs and employed a stepwise induction method with the addition of various factors in basal medium to successfully cultivate insulin-secreting β-cells (SC-β-cells), which functioned as fully functional pancreatic β-cells. Upon transplantation into mice, SC-β-cells showed detectable insulin secretion within two weeks, with secretion levels changing in response to blood glucose levels (42, 43). Directed differentiation protocols have also been reported for iPSCs, enabling the generation of cells expressing insulin and other mature β-cell markers (44, 45).

Because ESCs/iPSCs have good proliferation and differentiation ability and can produce large numbers of cells, they are ideal candidates for differentiation into islet β-cells and have broad application prospects for treating T1D. Therefore, how to produce SC-β-cells *in vitro* in large quantities has become the focus of research. The key transcription factors for differentiating ESCs/iPSCs into SC-β-cells *in vitro* are PDX1 and NKX6.1, both of which are highly expressed in pancreatic progenitor cells and required for producing monohormone, glucose-reactive β-cells (46, 47). Several research teams have reported differentiating ESCs/iPSCs into PDX1 and NKX6.1 co-expressing pancreatic progenitor cells (48, 49) in monolayer culture, and with improved experimental conditions, up to 90% of PDX1+/NKX6.1+ co-positive pancreatic progenitor cells were produced. Simultaneously, differentiating pancreatic progenitor cells into pancreatic β-cells has also made substantial progress, and the efficiency of pancreatic progenitor cells producing β-cells *in vitro* increased to about 40% (21, 42), although these β-cells are still different from human β-cells in other functions despite being responsive to glucose. To further improve the function of SC-beta cells, multiple research teams provided insights, such as Juan et al., who found that co-culturing with factors regulating circadian rhythm could enhance SC-beta cell function (50), Leonardo by altering the signaling pathway of SC-β-cells differentiation process (51), while Aharon et al. modified the nutrients in the medium used for *in vitro* differentiation to further enhance generating functional SC-β-cells *in vitro* (52), and could also induce SC-β-cells by simulating the 3D culture system of human pancreatic development (53). In addition, Isaura (54) and Mariana (55) et al. recently updated and detailed the recent progress in using ESCs/iPSCs derived islet β-cells *in vitro*. Some of the above protocols, although not reaching the level of the original human islet β-cells, promoted the development of stem cells differentiating into islet β-cells *in vitro*.

With the continuous development of stem cell technology, pancreatic β-cells products derived from ESCs/iPSCs are gradually used in clinical trials. A clinical trial conducted in 2014 (NCT02239354) used a stem cell-derived pancreatic endoderm cell population (PEC-01) developed by ViaCyte, Inc., which matured into insulin-producing endocrine cells *in vivo* over several months in animal models (56–58)., and in the clinical trials they have developed an immune protective device (PEC - Encap, VC - 01) is used to encapsulate PEC - 01, the device is a kind of biological membranes in order to eliminate the need for immunosuppression. The results of the trial showed that PEC-01 cell population could differentiate into β-cells and other islet cells after implantation under the patient's skin, but excessive fibrosis around the device resulted in the end of the trial due to immune rejection (29, 30). To address this problem, the new device was modified with an opening in the biofilm that allowed vascularization, enhanced nutrient exchange so that host cells could also penetrate the device, and immunosuppressive therapy was administered after transplantation, while a more mature and functional cell population (PEC-02) was used. The results of a subsequent clinical trial (NCT03163511), published in 2021, showed that transplanted cells matured from pancreatic progenitor cells to pancreatic endocrine cells six months after transplantation, producing glucose-reactive C-peptide (59, 60) in six of the 17 patients who underwent the trial. Although the circulating C-peptide levels observed in these studies are still low, all demonstrate the potential of ESCs/iPSCs to differentiate into renewable islet β-cells. Most importantly, both studies, although in early stage clinical studies, did not identify any serious safety issues related to the transplanted cells, including tumor formation. ViaCyte was later acquired by Vertex. Another direction of clinical trials is transplanting fully differentiated SC-β-cells, which have been successful in non-human primates (61, 62). The most promising clinical trial to date is Vertex's Phase I/II trial in 2021 (NCT04786262), which uses cells made of fully differentiated islet cells derived from pluripotent stem cells (VX-880) injected into the liver via a traditional portal route. Immunosuppressive therapy was also used to protect the transplanted islet cells from immune rejection. Some early results from the trial were recently published, with significant circulating C-peptide levels detected three months after transplantation and patients' blood sugar significantly controlled, And well tolerated treatment (63). VX-880 is a novel stem cell derived product for the treatment of T1D, and the trial is continuing in the United States and Canada to further evaluate the safety and efficacy of the product. As the technology develops, more clinical trials are expected.

In addition to using ESC/iPSC-based techniques to induce the differentiation of transplantable β-cells *in vitro*, Zeng et al. proposed an alternative solution. Using single-cell sequencing technology, they discovered a previously unreported cell population in the mouse pancreas: protein C receptor-positive (Procr+) pancreatic cell population. These Procr+ endocrine progenitor cells can be cultured and induced to differentiate into islet-like cells. In a transplantation model of diabetic mice, transplanted islet-like organs reversed the disease (36). This finding provides a new direction for the direct extraction of target cells from the pancreas and induction of their differentiation into islet-like organs.

3.2 Islet cells of porcine islet origin

In addition to using stem cell-derived islet beta cells to replace donor islet cells, another potential option is xenotransplantation using porcine islets. Compared to human islets, pig reproduction is

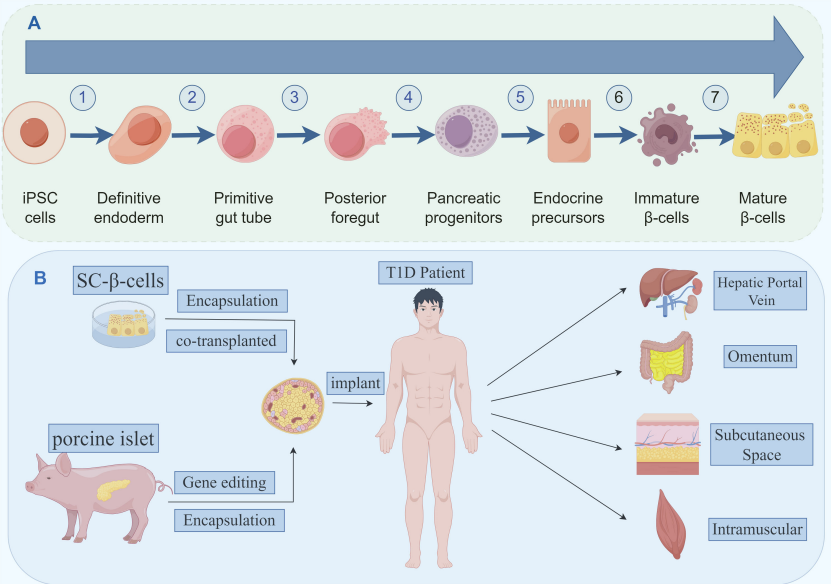


FIGURE 2
(A) The seven stages of differentiation of iPSCs into mature β-like cells. **(B)** Stem cell-derived beta cells and porcine islet-derived beta cells, which can be modified by a number of techniques and transplanted to potential transplantation sites.

easier and pig islets are more readily available. More importantly, pig insulin is highly similar to human insulin, differing by only one amino acid. Pig insulin has been used to treat diabetes for decades. Pigs have organs similar in size to humans, enabling production of a sufficient number of islets for xenotransplantation. They are the most promising donor source for xenotransplantation. Although more porcine islet cells are required to achieve adequate insulin secretion compared to human donor islets, porcine islets appear to outperform human islets in studies. Porcine islet cell xenotransplantation has achieved insulin function in non-human primates, suggesting feasibility in clinical settings. Shin et al. reported long-term survival of adult porcine islets transplanted into five rhesus monkeys for over 20 months. These early trials suggest pig islets have great potential to address donor islet shortages for T1D patients.

However, there are still some urgent problems to be solved in the use of porcine islet xenotransplantation, the first of which is graft rejection. For example, infusion of porcine islets into the portal vein leads to activation of complement and clotting pathways, resulting in platelet aggregation and thrombosis at the transplant site and hyperacute rejection (64). This is followed by human responses to porcine islet antigens (Galactose α1,3-galactose and N-Glycolylneuraminic acid), as well as zoonotic infections caused by endogenous retroviruses.

In the meantime, solutions are being tried. One strategy is encapsulating islet cells without immunosuppression to solve the immune rejection problem in porcine islet xenotransplantation. Various natural or synthetic biomaterials are used for encapsulation, such as polyethylene glycol diacrylate (PEG-DA) (65), agarose (66), and other biological materials like alginate (67). Coating islet cells with alginate films containing polyethylene glycol acrylate has allowed survival up to 6 months without immunosuppression (68, 69). However, encapsulation risks hypoxia and nutrient deficiency in islet cells, delayed glucose and insulin diffusion affecting glucose regulation (70). One possible immunosuppression approach is co-stimulatory blocking. Studies in non-human primates showed anti-CD154 monoclonal antibodies combined with stimulus-blocking and standard immunization regimens injected through the portal vein prolonged transplanted porcine mice survival. However, no clinically available anti-CD154 monoclonal antibodies exist due to high thrombosis risk (71). We summarized relevant studies using immunosuppressive therapy to prolong porcine islet survival post-transplantation in Table 2. Technological developments like gene editing technologies like CRISPR/Cas9 potentially eliminate endogenous viruses in pigs, improving porcine islet xenotransplantation safety to humans (31). Gene editing overexpresses or knocks out multiple genes finding the best transgenic pigs for islet transplantation, avoiding xenotransplantation rejection (72). Recent studies showed targeted controlled mutational events successfully generated in pig cells through nuclease-directed homologous recombination (32).

In general, various differentiation protocols are available to induce the transformation of ESCs/iPSCs into insulin-producing cells. Clinical trial results have shown its safety and tolerance, making it a hot topic in current research with broad application prospects. However, the approach of directly selecting cells from the pancreatic tissue to induce pancreatic-like organs should not be abandoned. Finally, although extensive data on pig islets are still required from nonhuman primates for safety validation before clinical trials, they have gained popularity among many researchers. These different sources of β-cell replacement provide abundant choices for future clinical applications, allowing personalized treatment plans based on individual patient conditions. We summarized the advantages and disadvantages of using ESCs/iPSCs derived islet β-cells and porcine islet instead of β-cells as shown in Table 3.

4 Ongoing challenges of islet transplantation immunosuppression

One of the greatest challenges that currently exists with islet transplantation is the post-transplant-induced recipient immune rejection, which may be responsible for the progressive decline in islet function in the years following islet transplantation as well as the inability of some patients to completely wean themselves from exogenous insulin therapy. These immune reactions include, but are not limited to: blood-mediated immediate inflammatory response (IBMIR) (73), recurrent autoimmune reactions (74, 75), and allogeneic rejection (76–78). Therefore, there is a clinical need to use high-quality islets from multiple donors or multiple inputs to counteract the substantial cell loss that occurs after transplantation (79). Currently, in order to overcome immune rejection after islet transplantation, in addition to the application of immunosuppressive drug, other new options have been explored, the most promising of which include the combined transplantation of mesenchymal stem cells (MSCs)/regulatory T cells (Tregs) and islet cells as well as the application of islet encapsulation techniques (Figure 3).

4.1 MSCs/Tregs were co-transplanted with islet cells

Mesenchymal stem cells (MSC),also known as stromal cells or mesenchymal progenitor cells, are a kind of non-hematopoietic stem

TABLE 3 Comparison between SC-β-cells and porcine islets for the imminent cure of T1D.

Type	Advantages	Disadvantages
SC-β-cells	• The ability to proliferate and differentiate indefinitely • Easy to genetically engineer • Potential for standardized industrial production • Encapsulation reduces immune rejection	• Difficulty *in vitro* differentiation • Lower functional performance of stem cell-derived islet cells compared to primary human islets • Use of different pluripotent stem cell lines and protocols
Porcine islets	• Easy access to islet donors • Functionally similar to human pancreatic islets • Successful trials in non-human primates • Encapsulation to render long-term function	• Immune rejection due to xenotransplantation • Zoonotic infections caused by endogenous retroviruses • Porcine islet antigen

cells derived from mesoderm, with multi-directional differentiation potential and strong self-renewal ability (80). MSC is relatively easy to obtain, can be obtained from human and rodent peripheral blood, placental tissue, umbilical cord blood, bone marrow cavity tissue and adipose tissue and other tissues and organs, and can be expanded and induced to differentiate *in vitro*, so it has been widely concerned and applied in the field of tissue engineering and regeneration. MSCs can improve the efficacy of IT in animal models, especially in regulating immune responses and protecting islet transplants (81–83). MSCs can improve insulin resistance in peripheral tissues through potential immunomodulatory and anti-inflammatory effects and promote pancreatic β-cell regeneration and protection (84, 85). Multiple studies have shown that, when co-cultured or co-transplanted with islet cells, MSCs can protect islet cells from apoptosis due to hypoxia and inflammatory cytokines through their secretory function, thus improving the survival of islet grafts *in vivo* and promoting the early recovery of the islet function (86, 87). In 2021, Kenyon et al. reported that islet cells and MSCs could be co-transplanted in non-human primate IT experiments. The results showed that the rejection-free survival and overall survival of treated islet grafts were significantly extended (88). Wang et al. used engineered MSCs as helper cells for islet co-transplantation and obtained similar results in diabetic mice. MSCs can induce local immune regulation and are potentially suitable for IT (89). Another study in patients with chronic pancreatitis showed that co-transplantation of autologous MSCs and islets is a safe and potential strategy for improving the islet function after transplantation (90). Generally speaking, co-transplantation with islet cells, it was found that mesenchymal stem cells had the functions of nutrition, support and protection to islet β-cells, as well as anti-inflammatory and immune regulation.

Regulatory T cells constitute a subset of T cells characterized by the presence of typical biological markers such as CD4+CD25+FoxP3+. These cells wield potent immunomodulatory functions and are pivotal in regulating immune homeostasis, upholding self-tolerance, and preventing excessive activation of the immune system (91). Tregs are considered a promising alternative to pharmacological agents that promote the engraftment and survival of transplanted organs/tissues (92–94). Tregs mainly produce self-tolerance, tolerance to alloantigens, and transplantation tolerance by inhibiting the activation and function of reactive effector T-cells (94). Currently, Treg therapy can be applied in two situations in IT: to promote the survival of islets during the initial transplantation and to induce peripheral tolerance to eliminate immunosuppression. The addition of Tregs at the time of islet infusion has been explored as a method to reduce the initial islet graft loss and improve islet engraftment (95–97). It has been reported that, in clinical models, Treg expansion *in vitro* and subsequent reinjection into patients can induce long-term remission of T1DM (98, 99). Although there are few relevant reports, a large amount of preclinical evidence shows that Treg-based treatment has benefits (100–102). Zielinski et al. recently reported a two-year study using a combined infusion of Tregs and rituximab to treat pediatric patients with T1DM. The study results show that combination therapy can delay disease progression compared with Treg or rituximab alone, and patients who received combination therapy were able to maintain higher insulin sensitivity and fasting C-peptide levels than patients in the single-treatment and control groups. Furthermore, patients who received Tregs alone had higher C-peptide levels than those in the untreated control group. Another ongoing clinical trial (NCT03182426) is observing the benefits of T cell depletion and dual anti-inflammatory treatment. If successful, it will provide new benefits to islet transplant patients.

With the development of IT, most traditional immunosuppressive drugs require continuous medication and cannot completely solve the problem of immune rejection in islet transplants. Cell therapy co-transplanted with MSCs/Tregs and islets has shown great advantages, although it is still in the experimental stage, and its application scenarios are broad.

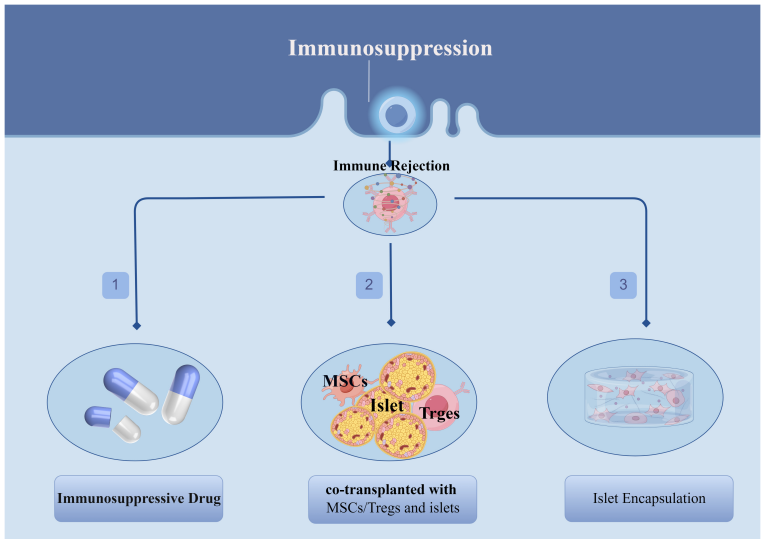


FIGURE 3
Different protocols for dealing with immune rejection after islet transplantation.

4.2 Islet encapsulation

Islet encapsulation represents a promising approach to tackle host immune rejection, employing biomaterials to envelop islets in a protective barrier. This allows oxygen and nutrients to permeate islet cells while enabling secreted insulin to disseminate into the bloodstream. Concurrently, it shields islet cells from assault by the host immune system (103–105). This technology has developed rapidly over the past century and can be categorized into micro and macro-encapsulation based on different processes.

Micro-encapsulation technology encapsulates islets in a thin layer of biomaterials, facilitating exchange of nutrients, oxygen, and metabolites. Transplantation of these micro-encapsulated islets is also simplified. Alginate stands out as a particularly promising biomaterial due to its superior biocompatibility and ease of manufacture. Studies confirm alginate reduces post-transplantation immune rejection and enhances survival of encapsulated islet cells (105). For instance, incorporating chemokine CXCL12 into alginate micro-encapsulation protects islets and boosts islet cell function even without immunosuppressants (106). This alginate-based micro-encapsulation method has also been applied to encapsulate SC-β-cells, exhibiting no excessive fibrosis post-transplantation sans immunosuppressive therapy (107). It has emerged as a key biomaterial for β-cell encapsulation studies. Recently, research teams have modified extracellular matrix (ECM) components into alginate, simulating the pancreatic microenvironment to safeguard coated islet cells from immune cell and inflammatory factor impacts while promoting insulin secretion by islet β-cells (108, 109). Nevertheless, several challenges persist in leveraging micro-encapsulation, especially post-implantation, presenting potential issues.

Another macro-encapsulation technique can prevent direct graft-host immune cell contact and spread, and enable easy removal of any post-transplantation safety issues, and evaluating graft efficacy at any time, unavailable with micro-encapsulation (110). Macro-encapsulation has combated host immune rejection but is limited by inadequate oxygen and nutrient exchange before blood vessel formation around the device (30). Adding vascular endothelial growth factor (VEGF) and pre-vascularization improved this (111, 112). Recently, Wang et al. developed a new device with immunoprotective hydrogel and thermoplastic silica gel-polycarbonate-polyurethane maintaining islet function for up to 200 days (113) in allogeneic rodent islet transplant models. Another macro-encapsulation type encapsulated SC-β cells with amphoteric modified alginate gel, reversing hyperglycemia for 238 days (114) post-implantation in severe combined immunodeficiency (SCID) mice. Many research teams are studying islet packaging, and we summarize recent progress in Table 4.

4.3 Optimal transplant site

Currently, most clinical IT methods involve injecting islet cells through the hepatic portal vein under ultrasound guidance. This is a conventional, mature method (11, 121). However, portal vein IT can cause postoperative bleeding, vascular emboli formation, portal hypertension, and periportal fatty degeneration. In particular, the blood-mediated acute inflammatory response (IBMIR) caused by portal vein transplantation can result in massive graft loss in the very early stages of transplantation (122), suggesting that the liver is not the most suitable site for IT. Researchers are exploring different organs and sites (Figure 2B) to determine the best location for islet cell transplantation (Table 5).

The omentum represents a potentially valuable transplant site, offering avoidance of IBMIR compared to traditional portal vein inflow. This richly vascularized tissue secretes various growth factors (e.g. CXCR4, VEGF, and SDF-1) that promote islet vascularization and survival (131, 132). In addition, omentum possesses immunomodulatory capabilities and can monitor the graft for prompt removal if adverse reactions occur. Omental transplantation using biological scaffolds has been used for clinical applications. A US trial (NCT02213003) transplanted pancreatic islets into the omentum of T1DM patients (133). Insulin independence was achieved by day 17 post-transplant but declined approximately one year later. Another ongoing trial (NCT02821026) has shown limited success. However, in 2023, Deng et al. reported a method of omental allogeneic IT in nonhuman primates using locally applied recombinant thrombin (Recothrom) and the recipient's autologous plasma to design a degradable matrix for islet fixation. Normal blood sugar and insulin independence were achieved at one week post-transplant, with stable expression thereafter. This study provides strategies for the clinical translation of omental transplantation.

The subcutaneous space is another ideal transplant site. It is a relatively avascular region that is easily accessible to biomaterials or macroscopically encapsulated islets. In 2020, Yu et al. reported successful subcutaneous IT in various immune-competent and immune-naïve animal models using a device-free islet survival matrix to achieve long-term normoglycemia. This method has been used for mice, pigs, and humans. Islet cell transplant models have the advantages of simplicity, safety, and reproducibility (134). With the clinical application of ESCs/iPSC-derived islet-like cells and islet encapsulation technology, the subcutaneous cavity can be easily monitored and removed, making it a promising transplant method. However, the skin lacks relative blood vessels and cannot obtain early-stage nutrients and oxygen, which limits its clinical application. To address this, Darling et al. tested a biodegradable temporary matrix based on a polyurethane scaffold that forms good blood vessels within the skin. In a porcine islet transplant model, grafts maintained normal function and survived for over three months (128). In addition, the immune response hinders subcutaneous transplantation. Therefore, the development of advanced biomaterials with angiogenesis and immune modulation capabilities may be the next step for the long-term islet survival and function in the skin.

In addition to the two aforementioned research hotspots of transplant sites, studies on transplanting islets into the intrapleural (135), skeletal muscle (136), anterior chamber of the eye (ACE) (137), and other sites have been reported (138–140). However, research on these aspects is still in its infancy, and there is a large gap in clinical applications. Due to the application of bioengineering materials and macro-encapsulated islet grafts, the greater omentum and subcutaneous space seem to be ideal sites for IT in the future.

TABLE 4 Different strategies and biomaterials for islet encapsulation.

Encapsulation material	Result	References
Carboxymethyl cellulose coated chitosan (CS@CMC) microgels	Long-term glucose regulation for 180 days was achieved in post-transplant diabetic mice	(115)
Methacrylated gelatin (GelMA), methacrylated heparin (HepMA) and VEGF	Reversed blood sugar levels in diabetic mice from high to normal blood sugar for at least 90 days	(116)
Zwitterionically modified alginate hydrogel	Hyperglycemia was reversed in SCID mice for 238 days	(114)
Immunoprotective hydrogel core and thermoplastic silicone-polycarbonate-urethane	In an allogeneic rodent islet transplantation model, use of the device was shown to maintain islet function for up to 200 days	(113)
Polytetrafluorethylene (PTFE)-membrane	Exhibit a rapid, vaso-independent and glucose-stimulated insulin response, early improvement of hyperglycemia and reduced fibrosis	(117)
Silicon nanopore membranes	Islets encapsulated with this device exhibit a highly active and biphasic insulin response to dynamic glucose stimulation	(118)
PTFE	After implantation, the patient experienced increased fasting C-peptide levels, increased glucose-reactive C-peptide levels, and mixed diet-stimulated C-peptide secretion.	(59)
Polyethylene glycol diacrylate (PEGDA)	The absence of immunosuppression reverses the signs of diabetes and leads to insulin-independent status or significantly reduced insulin requirements	(119)
Polyethylenglycol (PEG)	Reverse diabetes and maintain normal blood sugar for more than 80 days	(120)

4.4 Immunosuppression

Although several of the above options are effective in mitigating the immune rejection caused by islet transplantation and are the way forward, immunosuppressive therapy is still required at this time to ensure islet survival and function. The goal of immunosuppression is to provide effective and sustained immune protection in the smallest effective amount without suffering from the side effects associated with immunosuppression. Since inflammation leads to significant islet loss, anti-inflammatory drugs reduce damage from pro-inflammatory factors and may improve islet cell function in the early post-transplant period (141). Therefore, in order to attenuate the IBMIR response that occurs after islet transplantation and thereby reduce islet loss, several anti-inflammatory therapies have been used in the perioperative period of islet transplantation, including TNF-α inhibitors (etanercept), IL-1 receptor antagonists (anabolic acid), and α1-antitrypsin. Enalcipro, which targets TNF-α, is a potent antitumor agent that is widely used in T1D patients with allogeneic transplantation (17), and its use in mouse animal models results in a reduction of inflammatory markers and has been shown to have a sustained effect on autoimmunity (142). And in another study in an immunodeficient mouse islet transplant model, it was found that the percentage of mice achieving normal blood glucose levels after transplantation with the combination of etanercept and anabolic acid was 87.5%, compared to 45.45% with etanercept alone, and 53.9% with anabolic acid alone, suggesting that the combined use of etanercept and anabolic acid significantly improves the function of islet grafts (143). However, a recent study showed that although the use of etanercept demonstrated better islet function in the pre-transplant period, this advantage was not found to be sustained at the subsequent 1- or 2-year follow-up, and therefore, different doses or prolonged use of etanercept need to be explored to benefit patients (144). Another promising anti-inflammatory is α1-antitrypsin, which is a serine protease inhibitor, has been shown in several preclinical studies in animal islet transplantation models to attenuate the IBMIR response and prevent islet cell apoptosis while inhibiting cytokine-induced islet inflammatory responses (145, 146).

5 Conclusion and outlook

In terms of long-term results of islet transplantation, this study has greatly advanced research in the treatment of diabetes, and optimized protocols for long-term efficacy of islet transplantation have demonstrated the superiority of this approach, eliminating the dependence on exogenous insulin in a significant proportion of patients, thus avoiding diabetes-related complications. However islet transplantation still faces challenges such as shortage of islet sources and immunosuppression. To address the shortage of islet donors, we highlight stem cell-derived pancreatic β-cells and porcine islets as future solutions. Where stem cells are differentiated *in vitro* to generate pancreatic β-cells are being investigated for more efficient differentiation protocols, cell culture expansion methods and islet encapsulation techniques to optimize production to provide protection against the patient's autoimmune response. Porcine islet xenotransplantation is becoming a reality and if successful will provide a constant supply of high quality islet donors, however, xenoantigens and strong immunosuppressive responses are currently the main challenges and gene editing using CRISPR-Cas9 is expected to bring a brighter future for porcine islet xenotransplantation. In addition to overcome the immunosuppression, islet encapsulation technology is currently being developed, and various encapsulation materials: natural or synthetic biomaterials are showing clear advantages in several preclinical and clinical trials, and although the ideal biocompatible material is still a matter of debate, it is undeniable that islet encapsulation technology provides a barrier to protect transplanted islets, and in the future it will be mainly useful in preventing hyperfibrosis, promoting local vascularization, and preventing the emergence of chronic immunosuppressive rejection.

TABLE 5 Selection of transplantation sites other than the liver.

Transplantation sites	Receptor	Bio-materials	Result	References
Omentum	Diabetic rats	Hydrogels	Transplanted pancreatic islets show high rates of peri-islet and intra-islet hemotransfusion and reverse diabetes	(123)
	T1D patient	Biocompatible Plasma-Thrombin Gel	Stable glycemic control over 9 months, but relapse after 1 year	(124)
	Lewis rats	Plasma-thrombin bioscaffold	Maintained normal blood glucose for 100 days post-transplant	(125)
Intramuscular	7 years old patient		Quality of life improves, but exogenous insulin is still needed	(126)
	Lewis rats		Significantly lower blood sugar levels after islet transplantation	(127)
Subcutaneous space	Diabetic mice	Biodegradable temporizing matrix	Porcine islet cells survive more than 100 days after transplantation and secrete C-peptide	(128)
	Diabetic mice	Methacrylic acid-polyethylene glycol	Reversal of diabetes by injection of 600 rodent islet equivalents for 70 days	(129)
Anterior Chamber of the Eye (ACE)	Baboon		Decreased exogenous insulin requirement, no serious adverse effects seen	(130)

MSCs/Tregs and islet cell co-transplantation shows a broader prospect, which can minimize the use of immunosuppressant and reduce the side effects of immunosuppressant once it is successfully applied. Since islet grafts do not survive long term after portal vein infusion, which suggests that this site is not the optimal site for islet transplantation, subcutaneous lumen and greater omentum based encapsulation device is a more attractive strategy in comparison. Not only does it provide a physical barrier that reduces the destruction of the transplanted islets by the body's immune cells, thereby improving islet survival and function. At the same time, this strategy can be adapted as needed, such as removing the device in the event of an adverse reaction, and this flexibility can also be applied to individualize treatment as the patient's specific needs evolve.

The recent advent of single-cell sequencing technology (scRNA-seq) has ushered in a new era of molecular dissection, which is capable of revealing differential gene expression at the level of individual cells (147). In the field of islet transplantation, scRNA-seq may help to reveal the characteristics of different cell types in allogeneic islet transplants and be able to pinpoint cellular stress responses and pathophysiological changes in different grafts, which may further prolong islet graft survival and functional improvement, ultimately leading to insulin independence (148). In conclusion, with the innovative research carried out on islet source acquisition, immunosuppression protocols, and graft site reselection for islet transplantation, this technology will certainly be driven to greater maturity.

Author contributions

QW: Writing – original draft. Y-xH: Writing – original draft. LL: Writing – original draft. X-hZ: Writing – original draft. YS: Writing – original draft. XM: Writing – review & editing. S-wL: Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by Medical Science and Technology Project of Zhejiang Province (2024KY1788), Major Research Program of Taizhou Enze Medical Center Grant (19EZZDA2), Program of Taizhou Science and Technology Grant (23ywa33), Open Project Program of Key Laboratory of Minimally Invasive Techniques and Rapid Rehabilitation of Digestive System Tumor of Zhejiang Province (21SZDSYS01).

Conflict of interest

YS was employed by MSD China.

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.

Publisher's note

References

1. Atkinson MA, Eisenbarth GS, Michels AW. Type 1 diabetes. *Lancet*. (2014) 383:69–82. doi: 10.1016/S0140–6736(13)60591–7

2. Tripathi BK, Srivastava AK. Diabetes mellitus: complications and therapeutics. *Med Sci Monit*. (2006) 12:RA130–47.

3. Bergenstal RM, Tamborlane WV, Ahmann A, Buse JB, Dailey G, Davis SN, et al. Effectiveness of sensor-augmented insulin-pump therapy in type 1 diabetes. *N Engl J Med*. (2010) 363:311–20. doi: 10.1056/NEJMoa1002853

4. Karges B, Binder E, Rosenbauer J. Complications with insulin pump therapy vs insulin injection therapy-reply. *JAMA*. (2018) 319:503–4. doi: 10.1001/jama.2017.20357

5. Shapiro AM, Pokrywczynska M, Ricordi C. Clinical pancreatic islet transplantation. *Nat Rev Endocrinol*. (2017) 13:268–77. doi: 10.1038/nrendo.2016.178

6. Marfil-Garza BA, Shapiro AMJ, Kin T. Clinical islet transplantation: Current progress and new frontiers. *J Hepatobiliary Pancreat Sci*. (2021) 28:243–54. doi: 10.1002/jhbp.891

7. Israni AK, Zaun D, Rosendale JD, Snyder JJ, Kasiske BL. OPTN/SRTR 2012 Annual Data Report: deceased organ donation. *Am J Transplant*. (2014) 14 Suppl 1:167–83. doi: 10.1111/ajt.12585

8. Israni AK, Skeans MA, Gustafson SK, Schnitzler MA, Wainright JL, Carrico RJ, et al. OPTN/SRTR 2012 annual data report: pancreas. *Am J Transplant*. (2014) 14 Suppl 1:45–68. doi: 10.1111/ajt.12580

9. Shapiro AM. Islet transplantation in type 1 diabetes: ongoing challenges, refined procedures, and long-term outcome. *Rev Diabetes Stud*. (2012) 9:385–406. doi: 10.1900/RDS.2012.9.385

10. Sutherland DE, Gores PF, Farney AC, Wahoff DC, Matas AJ, Dunn DL, et al. Evolution of kidney, pancreas, and islet transplantation for patients with diabetes at the University of Minnesota. *Am J Surg*. (1993) 166:456–91. doi: 10.1016/s0002–9610(05)81142–0

11. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med*. (2000) 343:230–8. doi: 10.1056/NEJM200007273430401

12. Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, et al. International trial of the Edmonton protocol for islet transplantation. *N Engl J Med*. (2006) 355:1318–30. doi: 10.1056/NEJMoa061267

13. Markmann JF, Deng S, Huang X, Desai NM, Velidedeoglu EH, Lui C, et al. Insulin independence following isolated islet transplantation and single islet infusions. *Ann Surg*. (2003) 237:741–50. doi: 10.1097/01.SLA.0000072110.93780.52

14. Posselt AM, Szot GL, Frassetto LA, Masharani U, Tavakol M, Amin R, et al. Islet transplantation in type 1 diabetic patients using calcineurin inhibitor-free immunosuppressive protocols based on T-cell adhesion or costimulation blockade. *Transplantation*. (2010) 90:1595–601. doi: 10.1097/TP.0b013e3181fe1377

15. Posselt AM, Bellin MD, Tavakol M, Szot GL, Frassetto LA, Masharani U, et al. Islet transplantation in type 1 diabetics using an immunosuppressive protocol based on the anti-LFA-1 antibody efalizumab. *Am J Transplant*. (2010) 10:1870–80. doi: 10.1111/j.1600-6143.2010.03073.x

16. Hering BJ, Kandaswamy R, Harmon JV, Ansite JD, Clemmings SM, Sakai T, et al. Transplantation of cultured islets from two-layer preserved pancreases in type 1 diabetes with anti-CD3 antibody. *Am J Transplant*. (2004) 4:390–401. doi: 10.1046/j.1600-6143.2003.00351.x

17. Hering BJ, Kandaswamy R, Ansite JD, Eckman PM, Nakano M, Sawada T, et al. Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. *JAMA*. (2005) 293:830–5. doi: 10.1001/jama.293.7.830

18. Gangemi A, Salehi P, Hatipoglu B, Martellotto J, Barbaro B, Kuechle JB, et al. Islet transplantation for brittle type 1 diabetes: the UIC protocol. *Am J Transplant*. (2008) 8:1250–61. doi: 10.1111/j.1600-6143.2008.02234.x

19. Matsumoto S, Takita M, Chaussabel D, Noguchi Shimoda H, Sugimoto M, K, et al. Improving efficacy of clinical islet transplantation with iodixanol-based islet purification, thymoglobulin induction, and blockage of IL-1β and TNF-α. *Cell Transplant*. (2011) 20:1641–7. doi: 10.3727/096368910X564058

20. Maffi P, Berney T, Nano R, Niclauss N, Bosco D, Melzi R, et al. Calcineurin inhibitor-free immunosuppressive regimen in type 1 diabetes patients receiving islet transplantation: single-group phase 1/2 trial. *Transplantation*. (2014) 98:1301–9. doi: 10.1097/TP.0000000000000396

21. Hering BJ, Ballou CM, Bellin MD, Payne EH, Kandeel F, Witkowski P, et al. Factors associated with favourable 5 year outcomes in islet transplant alone recipients with type 1 diabetes complicated by severe hypoglycaemia in the Collaborative Islet Transplant Registry. *Diabetologia*. (2023) 66:163–73. doi: 10.1007/s00125–022-05804–4

22. Nijhoff MF, Engelse MA, Dubbeld J, Braat AE, Ringers J, Roelen DL, et al. Glycemic stability through islet-after-kidney transplantation using an alemtuzumab-based induction regimen and long-term triple-maintenance immunosuppression. *Am J Transplant*. (2016) 16:246–53. doi: 10.1111/ajt.13425

23. Cardona K, Korbutt GS, Milas Z, Lyon J, Cano J, Jiang W, et al. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat Med*. (2006) 12:304–6. doi: 10.1038/nm1375

24. Hering BJ, Wijkstrom M, Graham ML, Hårdstedt M, Aasheim TC, Jie T, et al. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. *Nat Med*. (2006) 12:301–3. doi: 10.1038/nm1369

25. Thompson P, Cardona K, Russell M, Badell IR, Shaffer V, Korbutt G, et al. CD40-specific costimulation blockade enhances neonatal porcine islet survival in nonhuman primates. *Am J Transplant*. (2011) 11:947–57. doi: 10.1111/j.1600-6143.2011.03509.x

26. Thompson P, Badell IR, Lowe M, Turner A, Cano J, Avila J, et al. Alternative immunomodulatory strategies for xenotransplantation: CD40/154 pathway-sparing regimens promote xenograft survival. *Am J Transplant*. (2012) 12:1765–75. doi: 10.1111/j.1600-6143.2012.04031.x

27. Shin JS, Kim JM, Kim JS, Min BH, Kim YH, Kim HJ, et al. Long-term control of diabetes in immunosuppressed nonhuman primates (NHP) by the transplantation of adult porcine islets. *Am J Transplant*. (2015) 15:2837–50. doi: 10.1111/ajt.13345

28. Shin JS, Kim JM, Min BH, Yoon IH, Kim HJ, Kim JS, et al. Pre-clinical results in pig-to-non-human primate islet xenotransplantation using anti-CD40 antibody (2C10R4)-based immunosuppression. *Xenotransplantation*. (2018) 25(1):10.1111/xen.12356. doi: 10.1111/xen.12356

29. Agulnick AD, Ambruzs DM, Moorman MA, Bhoumik A, Cesario RM, Payne JK, et al. Insulin-producing endocrine cells differentiated *in vitro* from human embryonic stem cells function in macroencapsulation devices *in vivo*. *Stem Cells Transl Med*. (2015) 4:1214–22. doi: 10.5966/sctm.2015–0079

30. Dolgin E. Diabetes: encapsulating the problem. *Nature*. (2016) 540:S60–2. doi: 10.1038/540S60a

31. Yang L, Güell M, Niu D, George H, Lesha E, Grishin D, et al. Genome-wide inactivation of porcine endogenous retroviruses (PERVs). *Science*. (2015) 350:1101–4. doi: 10.1126/science.aad1191

32. Butler JR, Santos RMN, Martens GR, Ladowski JM, Wang ZY, Li P, et al. Efficient generation of targeted and controlled mutational events in porcine cells using nuclease-directed homologous recombination. *J Surg Res*. (2017) 212:238–45. doi: 10.1016/j.jss.2017.01.025

33. Hering BJ, Clarke WR, Bridges ND, Eggerman TL, Alejandro R, Bellin MD, et al. Phase 3 trial of transplantation of human islets in type 1 diabetes complicated by severe hypoglycemia. *Diabetes Care*. (2016) 39:1230–40. doi: 10.2337/dc15–1988

34. Marfil-Garza BA, Imes S, Verhoeff K, Hefler J, Lam A, Dajani K, et al. Pancreatic islet transplantation in type 1 diabetes: 20-year experience from a single-centre cohort in Canada. *Lancet Diabetes Endocrinol*. (2022) 10:519–32. doi: 10.1016/S2213–8587(22)00114–0

35. Chetboun M, Drumez E, Ballou C, Maanaoui M, Payne E, Barton F, et al. Association between primary graft function and 5-year outcomes of islet allogeneic transplantation in type 1 diabetes: a retrospective, multicentre, observational cohort study in 1210 patients from the Collaborative Islet Transplant Registry. *Lancet Diabetes Endocrinol*. (2023) 11:391–401. doi: 10.1016/S2213–8587(23)00082–7

36. Wang D, Wang J, Bai L, Pan H, Feng H, Clevers H, et al. Long-term expansion of pancreatic islet organoids from resident procr+ Progenitors. *Cell*. (2020) 180:1198–1211.e19. doi: 10.1016/j.cell.2020.02.048

37. Li W, Nakanishi M, Zumsteg A, Shear M, Wright C, Melton DA, et al. *In vivo* reprogramming of pancreatic acinar cells to three islet endocrine subtypes. *Elife*. (2014) 3:e01846. doi: 10.7554/eLife.01846

38. Rodriguez-Diaz R, Molano RD, Weitz JR, Abdulreda MH, Berman DM, Leibiger B, et al. Paracrine interactions within the pancreatic islet determine the glycemic set point. *Cell Metab*. (2018) 27:549–558.e4. doi: 10.1016/j.cmet.2018.01.015

39. Hart NJ, Powers AC. Use of human islets to understand islet biology and diabetes: progress, challenges and suggestions. *Diabetologia*. (2019) 62:212–22. doi: 10.1007/s00125–018-4772–2

40. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol*. (2006) 24:1392–401. doi: 10.1038/nbt1259

41. Rezania A, Bruin JE, Arora P, Rubin A, Batushansky I, Asadi A, et al. Reversal of diabetes with insulin-producing cells derived *in vitro* from human pluripotent stem cells. *Nat Biotechnol*. (2014) 32:1121–33. doi: 10.1038/nbt.3033

42. Pagliuca FW, Millman JR, Gürtler M, Segel M, Van Dervort A, Ryu JH, et al. Generation of functional human pancreatic β-cells *in vitro*. *Cell*. (2014) 159:428–39. doi: 10.1016/j.cell.2014.09.040

43. Vegas AJ, Veiseh O, Gürtler M, Millman JR, Pagliuca FW, Bader AR, et al. Long-term glycemic control using polymer-encapsulated human stem cell-derived β-cells in immune-competent mice. *Nat Med*. (2016) 22:306–11. doi: 10.1038/nm.4030

44. Pagliuca FW, Melton DA. How to make a functional β-cell. *Development*. (2013) 140:2472–83. doi: 10.1242/dev.093187

45. Rostovskaya M, Bredenkamp N, Smith A. Towards consistent generation of pancreatic lineage progenitors from human pluripotent stem cells. *Philos Trans R Soc Lond B Biol Sci*. (2015) 370:20140365. doi: 10.1098/rstb.2014.0365

46. Rezania A, Bruin JE, Xu J, Narayan K, Fox JK, O'Neil JJ, et al. Enrichment of human embryonic stem cell-derived NKX6.1-expressing pancreatic progenitor cells accelerates the maturation of insulin-secreting cells *in vivo*. *Stem Cells*. (2013) 31:2432–42. doi: 10.1002/stem.1489

47. Jennings RE, Berry AA, Kirkwood-Wilson R, Roberts NA, Hearn T, Salisbury RJ, et al. Development of the human pancreas from foregut to endocrine commitment. *Diabetes*. (2013) 62:3514–22. doi: 10.2337/db12–1479

48. Nostro MC, Sarangi F, Yang C, Holland A, Elefanty AG, Stanley EG, et al. Efficient generation of NKX6–1+ pancreatic progenitors from multiple human pluripotent stem cell lines. *Stem Cell Rep*. (2015) 4:591–604. doi: 10.1016/j.stemcr.2015.02.017

49. Cogger KF, Sinha A, Sarangi F, McGaugh EC, Saunders D, Dorrell C, et al. Glycoprotein 2 is a specific cell surface marker of human pancreatic progenitors. *Nat Commun*. (2017) 8:331. doi: 10.1038/s41467–017-00561–0

50. Alvarez-Dominguez JR, Donaghey J, Rasouli N, Kenty JHR, Helman A, Charlton J, et al. Circadian entrainment triggers maturation of human *in vitro* islets. *Cell Stem Cell*. (2020) 26:108–122.e10. doi: 10.1016/j.stem.2019.11.011

51. Velazco-Cruz L, Song J, Maxwell KG, Goedegebuure MM, Augsornworawat P, Hogrebe NJ, et al. Acquisition of dynamic function in human stem cell-derived β Cells. *Stem Cell Rep*. (2019) 12:351–65. doi: 10.1016/j.stemcr.2018.12.012

52. Helman A, Cangelosi AL, Davis JC, Pham Q, Rothman A, Faust AL, et al. A nutrient-sensing transition at birth triggers glucose-responsive insulin secretion. *Cell Metab*. (2020) 31:1004–1016.e5. doi: 10.1016/j.cmet.2020.04.004

53. Gonçalves CA, Larsen M, Jung S, Stratmann J, Nakamura A, Leuschner M, et al. A 3D system to model human pancreas development and its reference single-cell transcriptome atlas identify signaling pathways required for progenitor expansion. *Nat Commun*. (2021) 12:3144. doi: 10.1038/s41467–021-23295–6

54. Silva IBB, Kimura CH, Colantoni VP, Sogayar MC. Stem cells differentiation into insulin-producing cells (IPCs): recent advances and current challenges. *Stem Cell Res Ther*. (2022) 13:309. doi: 10.1186/s13287-022-03206-2

55. Karimova MV, Gvazava IG, Vorotelyak EA. Overcoming the limitations of stem cell-derived beta cells. *Biomolecules*. (2022) 12:810. doi: 10.3390/biom12060810

56. Kelly OG, Chan MY, Martinson LA, Kadoya K, Ostertag TM, Ross KG, et al. Cell-surface markers for the isolation of pancreatic cell types derived from human embryonic stem cells. *Nat Biotechnol*. (2011) 29:750–6. doi: 10.1038/nbt.1931

57. Rezania A, Bruin JE, Riedel MJ, Mojibian M, Asadi A, Xu J, et al. Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice. *Diabetes*. (2012) 61:2016–29. doi: 10.2337/db11–1711

58. Kroon E, Martinson LA, Kadoya K, Bang AG, Kelly OG, Eliazer S, et al. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells *in vivo*. *Nat Biotechnol*. (2008) 26:443–52. doi: 10.1038/nbt1393

59. Ramzy A, Thompson DM, Ward-Hartstonge KA, Ivison S, Cook L, Garcia RV, et al. Implanted pluripotent stem-cell-derived pancreatic endoderm cells secrete glucose-responsive C-peptide in patients with type 1 diabetes. *Cell Stem Cell*. (2021) 28:2047–2061.e5. doi: 10.1016/j.stem.2021.10.003

60. Shapiro AMJ, Thompson D, Donner TW, Bellin MD, Hsueh W, Pettus J, et al. Insulin expression and C-peptide in type 1 diabetes subjects implanted with stem cell-derived pancreatic endoderm cells in an encapsulation device. *Cell Rep Med*. (2021) 2:100466. doi: 10.1016/j.xcrm.2021.100466

61. Du Y, Liang Z, Wang S, Sun D, Wang X, Liew SY, et al. Human pluripotent stem-cell-derived islets ameliorate diabetes in non-human primates. *Nat Med*. (2022) 28:272–82. doi: 10.1038/s41591–021-01645–7

62. Liang Z, Sun D, Lu S, Lei Z, Wang S, Luo Z, et al. Implantation underneath the abdominal anterior rectus sheath enables effective and functional engraftment of stem-cell-derived islets. *Nat Metab*. (2023) 5:29–40. doi: 10.1038/s42255–022-00713–7

63. Vertex. Vertex announces positive day 90 data for the frst patient in the phase 1/2 clinical trial dosed with VX-880, a Novel investigational stem cell-derived therapy for the treatment of type 1 diabetes . Available online at: https://news.vrtx.com/press-release/vertex-announces-positive-day-90-data-frstpatient-phase-12-clinical-trial-dosed-vx?_ga=2.53361578.345811804.1646342387–705593813.1646342387.

64. Ekser B, Cooper DK. Overcoming the barriers to xenotransplantation: prospects for the future. *Expert Rev Clin Immunol*. (2010) 6:219–30. doi: 10.1586/eci.09.81

65. Cruise GM, Hegre OD, Lamberti FV, Hager SR, Hill R, Scharp DS, et al. *In vitro* and *in vivo* performance of porcine islets encapsulated in interfacially photopolymerized poly(ethylene glycol) diacrylate membranes. *Cell Transplant*. (1999) 8:293–306. doi: 10.1177/096368979900800310

66. Gazda LS, Vinerean HV, Laramore MA, Hall RD, Carraway JW, Smith BH. No evidence of viral transmission following long-term implantation of agarose encapsulated porcine islets in diabetic dogs. *J Diabetes Res*. (2014) 2014:727483. doi: 10.1155/2014/727483

67. Pasqua M, Pereira U, de Lartigue C, Nicolas J, Vigneron P, Dermigny Q, et al. Preclinical characterization of alginate-poly-L-lysine encapsulated HepaRG for extracorporeal liver supply. *Biotechnol Bioeng*. (2021) 118:453–64. doi: 10.1002/bit.27583

68. Matsumoto S, Abalovich A, Wechsler C, Wynyard S, Elliott RB. Clinical benefit of islet xenotransplantation for the treatment of type 1 diabetes. *EBioMedicine*. (2016) 12:255–62. doi: 10.1016/j.ebiom.2016.08.034

69. Gianello P. Macroencapsulated pig islets correct induced diabetes in primates up to 6 months. *Adv Exp Med Biol*. (2015) 865:157–70. doi: 10.1007/978–3-319–18603-0_10

70. Korsgren O. Islet encapsulation: physiological possibilities and limitations. *Diabetes*. (2017) 66:1748–54. doi: 10.2337/db17–0065

71. Bottino R, Knoll MF, Graeme-Wilson J, Klein EC, Ayares D, Trucco M, et al. Safe use of anti-CD154 monoclonal antibody in pig islet xenotransplantation in monkeys. *Xenotransplantation*. (2017) 24(1):10.1111/xen.12283. doi: 10.1111/xen.12283

72. Cooper DKC, Mou L, Bottino R. A brief review of the current status of pig islet xenotransplantation. *Front Immunol*. (2024) 15:1366530. doi: 10.3389/fimmu.2024.1366530

73. Cheng Y, Wang B, Li H, Zhao N, Liu Y. Mechanism for the instant blood-mediated inflammatory reaction in rat islet transplantation. *Transplant Proc*. (2017) 49:1440–3. doi: 10.1016/j.transproceed.2017.03.090

74. Pearson T, Markees TG, Serreze DV, Pierce MA, Wicker LS, Peterson LB, et al. Islet cell autoimmunity and transplantation tolerance: two distinct mechanisms? *Ann N Y Acad Sci*. (2003) 1005:148–56. doi: 10.1196/annals.1288.016

75. Rossini AA, Mordes JP, Greiner DL, Stoff JS. Islet cell transplantation tolerance. *Transplantation*. (2001) 72:S43–6.

76. Eich T, Eriksson O, Lundgren TNordic Network for Clinical Islet Transplantation. Visualization of early engraftment in clinical islet transplantation by positron-emission tomography. *N Engl J Med*. (2007) 356:2754–5. doi: 10.1056/NEJMc070201

77. Eich T, Eriksson O, Sundin A, Estrada S, Brandhorst D, Brandhorst H, et al. Positron emission tomography: a real-time tool to quantify early islet engraftment in a preclinical large animal model. *Transplantation*. (2007) 84:893–8. doi: 10.1097/01.tp.0000284730.86567.9f

78. Citro A, Cantarelli E, Piemonti L. Anti-inflammatory strategies to enhance islet engraftment and survival. *Curr Diabetes Rep*. (2013) 13:733–44. doi: 10.1007/s11892–013-0401–0

79. McCall M, Shapiro AM. Update on islet transplantation. *Cold Spring Harb Perspect Med*. (2012) 2:a007823. doi: 10.1101/cshperspect.a007823

80. Brown C, McKee C, Bakshi S, Walker K, Hakman E, Halassy S, et al. Mesenchymal stem cells: Cell therapy and regeneration potential. *J Tissue Eng Regener Med*. (2019) 13:1738–55. doi: 10.1002/term.2914

81. Borg DJ, Weigelt M, Wilhelm C, Gerlach M, Bickle M, Speier S, et al. Mesenchymal stromal cells improve transplanted islet survival and islet function in a syngeneic mouse model. *Diabetologia*. (2014) 57:522–31. doi: 10.1007/s00125–013-3109–4

82. Newton WC, Kim JW, Luo JZQ, Luo L. Stem cell-derived exosomes: a novel vector for tissue repair and diabetic therapy. *J Mol Endocrinol*. (2017) 59:R155–65. doi: 10.1530/JME-17–0080

83. Mou L, Wang TB, Wang X, Pu Z. Advancing diabetes treatment: the role of mesenchymal stem cells in islet transplantation. *Front Immunol*. (2024) 15:1389134. doi: 10.3389/fimmu.2024.1389134

84. Shen J, Cheng Y, Han Q, Mu Y, Han W. Generating insulin-producing cells for diabetic therapy: existing strategies and new development. *Ageing Res Rev*. (2013) 12:469–78. doi: 10.1016/j.arr.2013.01.001

85. Ortiz LA, Gambelli F, McBride C, Gaupp D, Baddoo M, Kaminski N, et al. Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc Natl Acad Sci U S A*. (2003) 100:8407–11. doi: 10.1073/pnas.1432929100

86. Hubber EL, Rackham CL, Jones PM. Protecting islet functional viability using mesenchymal stromal cells. *Stem Cells Transl Med*. (2021) 10:674–80. doi: 10.1002/sctm.20–0466

87. Schive SW, Mirlashari MR, Hasvold G, Wang M, Josefsen D, Gullestad HP, et al. Human adipose-derived mesenchymal stem cells respond to short-term hypoxia by secreting factors beneficial for human islets *in vitro* and potentiate antidiabetic effect *in vivo*. *Cell Med*. (2017) 9:103–16. doi: 10.3727/215517917X693401

88. Kenyon NS, Willman MA, Han D, Leeman RS, Rabassa A, Diaz WL, et al. Extended survival versus accelerated rejection of nonhuman primate islet allografts: Effect of mesenchymal stem cell source and timing. *Am J Transplant*. (2021) 21:3524–37. doi: 10.1111/ajt.16693

89. Wang X, Wang K, Yu M, Velluto D, Hong X, Wang B, et al. Engineered immunomodulatory accessory cells improve experimental allogeneic islet transplantation without immunosuppression. *Sci Adv*. (2022) 8:eabn0071. doi: 10.1126/sciadv.abn0071

90. Wang H, Strange C, Nietert PJ, Wang J, Turnbull TL, Cloud C, et al. Autologous mesenchymal stem cell and islet cotransplantation: safety and efficacy. *Stem Cells Transl Med*. (2018) 7:11–9. doi: 10.1002/sctm.17–0139

91. Charbonnier LM, Cui Y, Stephen-Victor E, Harb H, Lopez D, Bleesing JJ, et al. Functional reprogramming of regulatory T cells in the absence of Foxp3. *Nat Immunol*. (2019) 20:1208–19. doi: 10.1038/s41590-019-0442-x

92. Martin-Moreno PL, Tripathi S, Chandraker A. Regulatory T cells and kidney transplantation. *Clin J Am Soc Nephrol*. (2018) 13:1760–4. doi: 10.2215/CJN.01750218

93. Romano M, Fanelli G, Albany CJ, Giganti G, Lombardi G. Past, present, and future of regulatory T cell therapy in transplantation and autoimmunity. *Front Immunol*. (2019) 10:43. doi: 10.3389/fimmu.2019.00043

94. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol*. (2005) 6:345–52. doi: 10.1038/ni1178

95. Yoon IH, Chung H, Kim HJ, Nam HY, Shin JS, Kim YH, et al. Peri-graft porcine-specific CD4+ FoxP3+ regulatory T cells by CD40-CD154 blockade prevented the rejection of porcine islet graft in diabetic mice. *Xenotransplantation*. (2019) 26:e12533. doi: 10.1111/xen.12533

96. Gregori S, Casorati M, Amuchastegui S, Smiroldo S, Davalli AM, Adorini L. Regulatory T cells induced by 1 alpha,25-dihydroxyvitamin D3 and mycophenolate mofetil treatment mediate transplantation tolerance. *J Immunol*. (2001) 167:1945–53. doi: 10.4049/jimmunol.167.4.1945

97. Gagliani N, Jofra T, Valle A, Stabilini A, Morsiani C, Gregori S, et al. Transplant tolerance to pancreatic islets is initiated in the graft and sustained in the spleen. *Am J Transplant*. (2013) 13:1963–75. doi: 10.1111/ajt.12333

98. Grinberg-Bleyer Y, Baeyens A, You S, Elhage R, Fourcade G, Gregoire S, et al. IL-2 reverses established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells. *J Exp Med*. (2010) 207:1871–8. doi: 10.1084/jem.20100209

99. Tang Q, Henriksen KJ, Bi M, Finger EB, Szot G, Ye J, et al. *In vitro*-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med*. (2004) 199:1455–65. doi: 10.1084/jem.20040139

100. Gagliani N, Jofra T, Stabilini A, Valle A, Atkinson M, Roncarolo MG, et al. Antigen-specific dependence of Tr1-cell therapy in preclinical models of islet transplant. *Diabetes*. (2010) 59:433–9. doi: 10.2337/db09–1168

101. Lee K, Nguyen V, Lee KM, Kang SM, Tang Q. Attenuation of donor-reactive T cells allows effective control of allograft rejection using regulatory T cell therapy. *Am J Transplant*. (2014) 14:27–38. doi: 10.1111/ajt.12509

102. Pierini A, Iliopoulou BP, Peiris H, Pérez-Cruz M, Baker J, Hsu K, et al. T cells expressing chimeric antigen receptor promote immune tolerance. *JCI Insight*. (2017) 2: e92865. doi: 10.1172/jci.insight.92865

103. Chang TM. SEMIPERMEABLE MICROCAPSULES. *Science*. (1964) 146:524–5. doi: 10.1126/science.146.3643.524

104. Olabisi RM. Cell microencapsulation with synthetic polymers. *J BioMed Mater Res A*. (2015) 103:846–59. doi: 10.1002/jbm.a.35205

105. Mallett AG, Korbutt GS. Alginate modification improves long-term survival and function of transplanted encapsulated islets. *Tissue Eng Part A*. (2009) 15:1301–9. doi: 10.1089/ten.tea.2008.0118

106. Chen T, Yuan J, Duncanson S, Hibert ML, Kodish BC, Mylavaganam G, et al. Alginate encapsulant incorporating CXCL12 supports long-term allo- and xenoislet transplantation without systemic immune suppression. *Am J Transplant*. (2015) 15:618–27. doi: 10.1111/ajt.13049

107. Alagpulinsa DA, Cao JJL, Driscoll RK, Sîrbulescu RF, Penson MFE, Sremac M, et al. Alginate-microencapsulation of human stem cell-derived β cells with CXCL12 prolongs their survival and function in immunocompetent mice without systemic immunosuppression. *Am J Transplant*. (2019) 19:1930–40. doi: 10.1111/ajt.15308

108. Enck K, Tamburrini R, Deborah C, Gazia C, Jost A, Khalil F, et al. Effect of alginate matrix engineered to mimic the pancreatic microenvironment on encapsulated islet function. *Biotechnol Bioeng*. (2021) 118:1177–85. doi: 10.1002/bit.27641

109. Krishtul S, Skitel Moshe M, Kovrigina I, Baruch L, Machluf M. ECM-based bioactive microencapsulation significantly improves islet function and graft performance. *Acta Biomater*. (2023) 171:249–60. doi: 10.1016/j.actbio.2023.09.009

110. Goswami D, Domingo-Lopez DA, Ward NA, Millman JR, Duffy GP, Dolan EB, et al. Design considerations for macroencapsulation devices for stem cell derived islets for the treatment of type 1 diabetes. *Adv Sci (Weinh)*. (2021) 8:e2100820. doi: 10.1002/advs.202100820

111. Kasoju N, Pátíková A, Wawrzynska E, Vojtíšková A, Sedlačík T, Kumorek M, et al. Bioengineering a pre-vascularized pouch for subsequent islet transplantation using VEGF-loaded polylactide capsules. *Biomater Sci*. (2020) 8:631–47. doi: 10.1039/C9BM01280J

112. Wang LH, Marfil-Garza BA, Ernst AU, Pawlick RL, Pepper AR, Okada K, et al. Inflammation-induced subcutaneous neovascularization for the long-term survival of encapsulated islets without immunosuppression. *Nat BioMed Eng*. (2023), 10.1038/s41551-023-01145-8. doi: 10.1038/s41551–023-01145–8

113. Wang X, Maxwell KG, Wang K, Bowers DT, Flanders JA, Liu W, et al. A nanofibrous encapsulation device for safe delivery of insulin-producing cells to treat type 1 diabetes. *Sci Transl Med*. (2021) 13:eabb4601. doi: 10.1126/scitranslmed.abb4601

114. Liu W, Flanders JA, Wang LH, Liu Q, Bowers DT, Wang K, et al. A safe, fibrosis-mitigating, and scalable encapsulation device supports long-term function of insulin-producing cells. *Small*. (2022) 18:e2104899. doi: 10.1002/smll.202104899

115. Li H, He W, Feng Q, Chen J, Xu X, Lv C, et al. Engineering superstable islets-laden chitosan microgels with carboxymethyl cellulose coating for long-term blood glucose regulation *in vivo*. *Carbohydr Polym*. (2024) 323:121425. doi: 10.1016/j.carbpol.2023.121425

116. Li H, Shang Y, Feng Q, Liu Y, Chen J, Dong H. A novel bioartificial pancreas fabricated via islets microencapsulation in anti-adhesive core-shell microgels and macroencapsulation in a hydrogel scaffold prevascularized *in vivo*. *Bioact Mater*. (2023) 27:362–76. doi: 10.1016/j.bioactmat.2023.04.011

117. Yang K, O'Cearbhaill ED, Liu SS, Zhou A, Chitnis GD, Hamilos AE, et al. A therapeutic convection-enhanced macroencapsulation device for enhancing β cell viability and insulin secretion. *Proc Natl Acad Sci U S A*. (2021) 118:e2101258118. doi: 10.1073/pnas.2101258118

118. Shaheen R, Gurlin RE, Gologorsky R, Blaha C, Munnangi P, Santandreu A, et al. Superporous agarose scaffolds for encapsulation of adult human islets and human stem-cell-derived β cells for intravascular bioartificial pancreas applications. *J BioMed Mater Res A*. (2021) 109:2438–48. doi: 10.1002/jbm.a.37236

119. Harrington S, Karanu F, Ramachandran K, Williams SJ, Stehno-Bittel L. PEGDA microencapsulated allogeneic islets reverse canine diabetes without immunosuppression. *PloS One*. (2022) 17:e0267814. doi: 10.1371/journal.pone.0267814

120. Stock AA, Manzoli V, De Toni T, Abreu MM, Poh YC, Ye L, et al. Conformal coating of stem cell-derived islets for β Cell replacement in type 1 diabetes. *Stem Cell Rep*. (2020) 14:91–104. doi: 10.1016/j.stemcr.2019.11.004

121. Schmidt C. Pancreatic islets find a new transplant home in the omentum. *Nat Biotechnol*. (2017) 35:8. doi: 10.1038/nbt0117–8

122. Johansson H, Goto M, Dufrane D, Siegbahn A, Elgue G, Gianello P, et al. Low molecular weight dextran sulfate: a strong candidate drug to block IBMIR in clinical islet transplantation. *Am J Transplant*. (2006) 6:305–12. doi: 10.1111/j.1600-6143.2005.01186.x

123. Schaschkow A, Mura C, Pinget M, Bouzakri K, Maillard E. Intra-Omental Islet Transplantation Using h-Omental Matrix Islet filliNG (hOMING). *J Vis Exp*. (2019) 145):10. doi: 10.3791/58898

124. Saudek F, Hladiková Z, Hagerf B, Nemetova L, Girman P, Kriz J, et al. Transplantation of pancreatic islets into the omentum using a biocompatible plasma-thrombin gel: first experience at the institute for clinical and experimental medicine in prague. *Transplant Proc*. (2022) 54:806–10. doi: 10.1016/j.transproceed.2021.11.037

125. Hladiková Z, Voglová B, Pátíková A, Berková Z, Kříž J, Vojtíšková A, et al. Bioluminescence imaging *in vivo* confirms the viability of pancreatic islets transplanted into the greater omentum. *Mol Imaging Biol*. (2021) 23:639–49. doi: 10.1007/s11307-021-01588-y

126. Rafael E, Tibell A, Rydén M, Lundgren T, Sävendahl L, Borgström B, et al. Intramuscular autotransplantation of pancreatic islets in a 7-year-old child: a 2-year follow-up. *Am J Transplant*. (2008) 8:458–62. doi: 10.1111/j.1600-6143.2007.02060.x

127. Park JL, Kim T, Kim BK. Suitability of denervated muscle flaps as recipient sites for pancreatic islet cell transplantation. *Arch Plast Surg*. (2021) 48:133–43. doi: 10.5999/aps.2020.01865

128. Rojas-Canales D, Walters SN, Penko D, Cultrone D, Bailey J, Chtanova T, et al. Intracutaneous transplantation of islets within a biodegradable temporizing matrix as an alternative site for islet transplantation. *Diabetes*. (2023) 72:758–68. doi: 10.2337/db21–0841

129. Kinney SM, Ortaleza K, Vlahos AE, Sefton MV. Degradable methacrylic acid-based synthetic hydrogel for subcutaneous islet transplantation. *Biomaterials*. (2022) 281:121342. doi: 10.1016/j.biomaterials.2021.121342

130. Perez VL, Caicedo A, Berman DM, Arrieta E, Abdulreda MH, Rodriguez-Diaz R, et al. The anterior chamber of the eye as a clinical transplantation site for the treatment of diabetes: a study in a baboon model of diabetes. *Diabetologia*. (2011) 54:1121–6. doi: 10.1007/s00125-011-2091-y

131. Litbarg NO, Gudehithlu KP, Sethupathi P, Arruda JA, Dunea G, Singh AK. Activated omentum becomes rich in factors that promote healing and tissue regeneration. *Cell Tissue Res*. (2007) 328:487–97. doi: 10.1007/s00441–006-0356–4

132. Damyar K, Farahmand V, Whaley D, Alexander M, Lakey JRT. An overview of current advancements in pancreatic islet transplantation into the omentum. *Islets*. (2021) 13:115–20. doi: 10.1080/19382014.2021.1954459

133. Baidal DA, Ricordi C, Berman DM, Alvarez A, Padilla N, Ciancio G, et al. Bioengineering of an intraabdominal endocrine pancreas. *N Engl J Med*. (2017) 376:1887–9. doi: 10.1056/NEJMc1613959

134. Yu M, Agarwal D, Korutla L, May CL, Wang W, Griffith NN, et al. Islet transplantation in the subcutaneous space achieves long-term euglycaemia in preclinical models of type 1 diabetes. *Nat Metab*. (2020) 2:1013–20. doi: 10.1038/s42255–020-0269–7

135. Lei J, Zhang A, Deng H, Yang Z, Peters CW, Lee KM, et al. Intrapleural transplantation of allogeneic pancreatic islets achieves glycemic control in a diabetic non-human primate. *Am J Transplant*. (2022) 22:966–72. doi: 10.1111/ajt.16875

136. Zhang M, Du H, Guan Y, Liu J, Wang S, Li H, et al. Study on the effect of PDA-PLGA scaffold loaded with islet cells for skeletal muscle transplantation in the treatment of diabetes. *Front Bioeng Biotechnol*. (2022) 10:927348. doi: 10.3389/fbioe.2022.927348

137. Ilegems E, Berggren PO. The eye as a transplantation site to monitor pancreatic islet cell plasticity. *Front Endocrinol (Lausanne)*. (2021) 12:652853. doi: 10.3389/fendo.2021.652853

138. Cayabyab F, Nih LR, Yoshihara E. Advances in pancreatic islet transplantation sites for the treatment of diabetes. *Front Endocrinol (Lausanne)*. (2021) 12:732431. doi: 10.3389/fendo.2021.732431

139. Wagner LE, Melnyk O, Duffett BE, Linnemann AK. Mouse models and human islet transplantation sites for intravital imaging. *Front Endocrinol (Lausanne)*. (2022) 13:992540. doi: 10.3389/fendo.2022.992540

140. Li F, Lv Y, Li X, Yang Z, Guo T, Zhang J. Comparative study of two different islet transplantation sites in mice: hepatic sinus tract vs splenic parenchyma. *Cell Transplant*. (2020) 29:963689720943576. doi: 10.1177/0963689720943576

141. Szempruch KR, Banerjee O, McCall RC, Desai CS. Use of anti-inflammatory agents in clinical islet cell transplants: A qualitative systematic analysis. *Islets*. (2019) 11:65–75. doi: 10.1080/19382014.2019.1601543

142. Westwell-Roper C, Dai DL, Soukhatcheva G, Potter KJ, van Rooijen N, Ehses JA, et al. IL-1 blockade attenuates islet amyloid polypeptide-induced proinflammatory cytokine release and pancreatic islet graft dysfunction. *J Immunol*. (2011) 187:2755–65. doi: 10.4049/jimmunol.1002854

143. McCall M, Pawlick R, Kin T, Shapiro AM. Anakinra potentiates the protective effects of etanercept in transplantation of marginal mass human islets in immunodeficient mice. *Am J Transplant*. (2012) 12:322–9. doi: 10.1111/j.1600-6143.2011.03796.x

144. Abdel-Karim TR, Hodges JS, Herold KC, Pruett TL, Ramanathan KV, Hering BJ, et al. Peri-transplant inflammation and long-term diabetes outcomes were not impacted by either etanercept or alpha-1-antitrypsin treatment in islet autotransplant recipients. *Transpl Int*. (2024) 37:12320. doi: 10.3389/ti.2024.12320

145. Shahaf G, Moser H, Ozeri E, Mizrahi M, Abecassis A, Lewis EC. α-1-antitrypsin gene delivery reduces inflammation, increases T-regulatory cell population size and prevents islet allograft rejection. *Mol Med*. (2011) 17:1000–11. doi: 10.2119/molmed.2011.00145

146. Wang J, Sun Z, Gou W, Adams DB, Cui W, Morgan KA, et al. α-1 antitrypsin enhances islet engraftment by suppression of instant blood-mediated inflammatory reaction. *Diabetes*. (2017) 66:970–80. doi: 10.2337/db16–1036

147. Baysoy A, Bai Z, Satija R, Fan R. The technological landscape and applications of single-cell multi-omics. *Nat Rev Mol Cell Biol*. (2023) 24:695–713. doi: 10.1038/s41580-023-00615-w

148. Mohiuddin MM, Singh AK, Scobie L, Goerlich CE, Grazioli A, Saharia K, et al. Graft dysfunction in compassionate use of genetically engineered pig-to-human cardiac xenotransplantation: a case report. *Lancet*. (2023) 402:397–410. doi: 10.1016/S0140–6736(23)00775–4

Frontiers in Immunology

Explores novel approaches and diagnoses to treat immune disorders.

The official journal of the International Union of Immunological Societies (IUIS) and the most cited in its field, leading the way for research across basic, translational and clinical immunology.

Discover the latest Research Topics

See more →

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact