PROGRESS OF ALLO- AND XENO-TRANSPLANTATION FACILITATING THE INITIAL XENO-KIDNEY AND ISLET CLINICAL TRIALS

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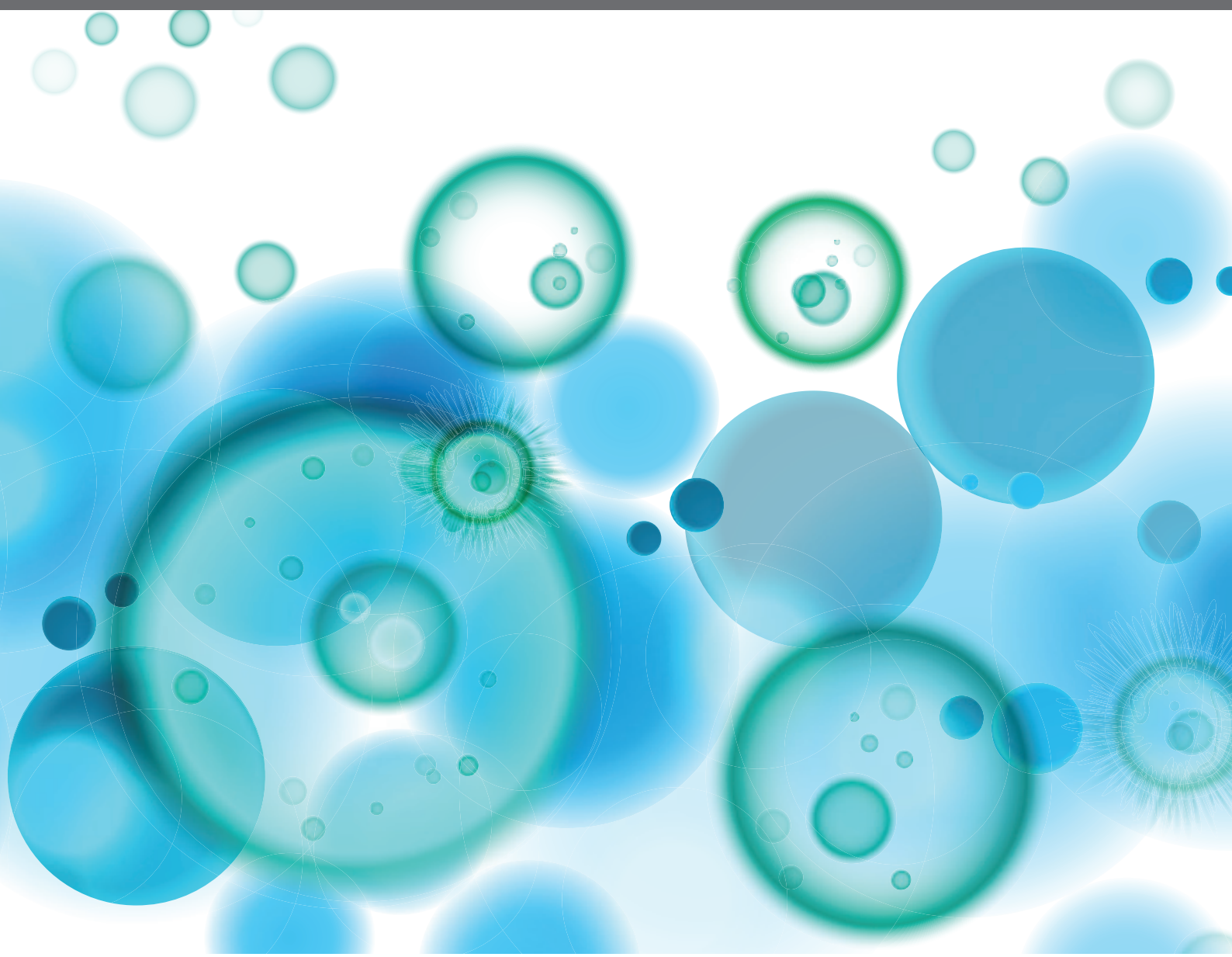

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PROGRESS OF ALLO- AND XENO-TRANSPLANTATION FACILITATING THE INITIAL XENO-KIDNEY AND ISLET CLINICAL TRIALS

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Table of Contents

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The Role of Interleukin-6 (IL-6) in the Systemic Inflammatory Response in Xenograft Recipients and in Pig Kidney Xenograft Failure

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Background: In pig-to-baboon transplantation models, there is increasing evidence of systemic inflammation in xenograft recipients (SIXR) associated with pig xenograft failure. We evaluated the relationship between systemic inflammatory factors and pig kidney xenograft failure.

Methods: Baboons received kidney transplants from genetically engineered pigs (n=9), and received an anti-CD40mAb-based (n=4) or conventional (n=5) immunosuppressive regimen. The pig kidney grafts were monitored by measurements of serum creatinine, serum amyloid A (SAA), white blood cell (WBC) and platelet counts, plasma fibrinogen, and pro-inflammatory cytokines (baboon and pig IL-6, TNF-α, IL-1β).

Results: Six baboons were euthanized or died from rejection, and 3 were euthanized for infection. Changes in serum creatinine correlated with those of SAA ($r=0.56$, $p<0.01$). Serum *baboon* IL-6 was increased significantly on day 1 after transplantation and at euthanasia (both $p<0.05$) and correlated with serum creatinine and SAA ($r=0.59$, $p<0.001$, $r=0.58$, $p<0.01$; respectively). but no difference was observed between rejection and infection. Levels of serum *pig* IL-6, TNF-α, IL-1β were also significantly increased on day 1 and at euthanasia, and serum pig IL-6 and IL-1β correlated with serum creatinine and SAA. The level of serum baboon IL-6 correlated with the expression of IL-6 and amyloid A in the baboon liver ($r=0.93$, $p<0.01$, $r=0.79$, $p<0.05$; respectively).

Conclusion: Early upregulation of SAA and serum IL-6 may indicate the development of rejection or infection, and are associated with impaired kidney graft function. Detection and prevention of systemic inflammation may be required to prevent pig kidney xenograft failure after xenotransplantation.

Keywords: baboon, po-inflammatory cytokines, inflammation, IL-6, kidney, pig, serum amyloid A, xenotransplantation

INTRODUCTION

Organ transplantation is the preferred method of treatment of end-stage organ failure, but a severe shortage of deceased human donor organs is a major limitation. Great progress has been made in the study of xenotransplantation since the first chimpanzee-to-human kidney xenotransplant was performed by Reemtsma in 1963. The pig has since been identified as a potential source of organs for clinical transplantation (1), but there remain several barriers in pig-to-primate organ xenotransplantation that have to be overcome. One of these is the systemic inflammatory response in the xenograft recipient (SIXR) to the presence of a pig organ (2–4).

Inflammation is a complex biological defensive response to infection or biological stress, in which pro-inflammatory cytokines activate the immune system. However, an excessive inflammatory response can result in various pathological states, such as coagulation and/or immune dysregulation (5). In kidney allotransplantation, inflammatory signals promote T cell activation and play an important role in rejection, impairing T cell tolerance and preventing long-term graft survival (6). In pig organ xenotransplantation, SIXR, as evidenced by a sustained rise in C-reactive protein (C-RP), precedes the development of a consumptive coagulopathy (7).

Kidney graft failure is largely documented by an increase in the serum creatinine, with confirmation by histopathologic assessment of kidney graft biopsies. We suggest that SIXR may be closely associated with pig kidney graft failure, and therefore the detection and prevention of an inflammatory response may play an important role in prolonging the survival of a pig xenograft in a nonhuman primate (NHP). C-RP is an acute phase protein that is synthesized by hepatocytes in response to proinflammatory cytokines, in particular interleukin-6 (IL-6) and is widely used to evaluate an inflammatory response. However, it can be significantly inhibited by IL-6 receptor blockade (e.g., with tocilizumab) (8–10). Serum amyloid A (SAA) is a more sensitive and convenient marker than C-RP when anti-inflammatory therapy is being administered (11–13). Other inflammatory markers, e.g., serum histones, free triiodothyronine, plasma fibrinogen, and platelet count, can help monitor the inflammatory response or health status of NHP recipients of pig xenografts (14, 15).

The pro-inflammatory cytokines, e.g., IL-6, TNF-α, and IL-1β, involved in the pathological processes of rejection and infection in baboon xenograft recipients (3, 4, 16, 17), and may be associated with coagulation dysfunction (7, 14). IL-6 increases to a high level in baboons after pig kidney xenotransplantation. *In vitro* studies, baboon IL-6 can activate pig aortic endothelial cells (18). However the roles of pro-inflammatory cytokines in SIXR-associated rejection or infection remain unclear.

We measured inflammatory factors in NHP recipients of pig kidney grafts, and provide evidence that SIXR is associated with pig kidney graft failure from rejection or with infection.

Abbreviations: C-RP, C-reactive protein; IL-6, interleukin-6; NHP, nonhuman primate; SAA, serum amyloid A; SIXR, systemic inflammation in xenograft recipients.

MATERIALS AND METHODS

Animals and Kidney Transplantation

Baboons (n=9) (Oklahoma University Health Sciences Center, Oklahoma City, OK) received a life-supporting kidney graft from genetically-engineered pigs (Revivicor, Blacksburg, VA) (**Table 1**). The operative procedures and management of the baboons have been detailed previously (19, 20).

All animal care was in accordance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985). All procedures were approved by the Institutional Animal Care and Use Committees of the University of Pittsburgh and the University of Alabama at Birmingham.

Immunosuppressive and Anti-Inflammatory Therapy

All baboons received thymoglobulin (ATG) and anti-CD20mAb (Rituximab) as induction therapy. Maintenance immunosuppressive regimens are summarized in **Table 1** (together with other information, e.g., graft survival and cause of death). The immunosuppressive regimens were either anti-CD40mAb-based or conventional therapy-based (which consisted of only FDA-approved agents – with various combinations of CTLA4-Ig, tacrolimus, rapamycin, mycophenolate mofetil) (21–23). Anti-CD40mAb (2C10R4, a chimeric rhesus IgG4) was provided by the NIH NHP Reagent Resource (24). Anti-inflammatory therapy was administered in an attempt to reduce inflammation. All baboons received interleukin-6 receptor (IL-6R) blockade with tocilizumab (10mg/kg on days –1, 7, 14, and thereafter every 2 weeks; Actemra, Genentech, South San Francisco, CA), and the TNF-αantagonist, etanercept (0.5mg/kg on days 0, 3, 7, and 10, Amgen, Thousand Oaks, CA).

Monitoring of Recipient Baboons

Whole blood and serum samples were obtained from recipients before and serially after transplantation for measurement of blood cell counts, chemistry (kidney function, etc.), coagulation parameters, and the inflammatory response (based on measurement of SAA and selected serum cytokines). Blood cell counts were measured by standard methods (Central Laboratory of Presbyterian Hospital, Pittsburgh, PA).

Serum Amyloid A

SAA was measured by the Rapid Test for Inflammation & Infection kit (Accuplex, Maynooth, Co Kildare, Ireland), as an indication of inflammation, per the manufacturer's instructions (13). The results of the SAA assay were categorized into groups, using the following scoring system (1=no or minimal inflammation; 2=moderate inflammation; 3=severe inflammation).

Serum Cytokine Assays

Serum cytokine assays were performed as previously described (18). The levels of cytokines (baboon IL-6, TNF-α, IL-1β) in baboon serum were measured by cytometric bead array (CBA)

TABLE 1 | Pig genetic engineering, immunosuppressive therapy, recipient survival, and causes of death with tocilizumab treatment.

Baboon Number	Donor pig genetic engineered phenotype	Maintenance IS therapy	Survival (days)	Cause of Death
Group A (Rejection)				
B14115	GTKO/CD46/TBM/EPCR/CD47/HO1	Conventional therapy	1	HAR
B10815	GTKO/CD46/TBM/EPCR/CD47/HO1	Anti-CD40mAb-based	90	AHXR
B14214	GTKO/CD46/hvWF	Conventional therapy	32	AHXR
B5415	GTKO/CD46/TBM/EPCR/CD47/HO1	Conventional therapy	15	AHXR
B9015	GTKO/CD46/TBM/EPCR/CD47/HO1	Conventional therapy	13	AHXR
B15815	GTKO/CD46/TBM/EPCR/CD47/HO1	Conventional therapy	12	AHXR
Group B (Infection)				
B9313	GTKO/CD46/CD55/TBM/EPCR/CD39	Anti-CD40mAb-based	136	infection
B17315	GTKO/CD46/CD55/EPCR/TFPI/CD47	Anti-CD40mAb-based	237	infection
B17615	GTKO/CD46/CD55/EPCR/TFPI/CD47	Anti-CD40mAb-based	260	infection

HAR, hyperacute rejection; AHXR, acute humoral xenograft rejection; GTKO, α1,3-galactosyltransfearse gene-knockout; TBM, thrombomodulin; EPCR, endothelial protein C receptor; HO1, heme oxygenase-1; hvWF, human von Willebrand factor; TFPI, tissue factor pathway inhibitor. Conventional therapy consisted of only FDA-approved agents (various combinations of CTLA4-Ig, tacrolimus, rapamycin, mycophenolate mofetil).

with a human Inflammatory Cytokine Kit (No. 551811; BD Biosciences, San Jose, CA), according to the manufacturer's instructions. LSR II flow cytometry (BD Biosciences) was used to collect data, which were analyzed using CBA analysis software (BD Biosciences). Levels of pig IL-6 in baboon serum were measured by ELISA using a porcine IL-6 Quantikine ELISA Kit (No. P6000B; R&D Systems, Minneapolis, MN), following the manufacturer's instructions. Optical density was measured by using a Wallac Victor3 1420 Multilabel Counter (Perkin Elmer, Waltham, MA) at 450nm, with the correction wavelength set at 540nm or 570nm.

Immunohistochemistry

Baboon liver tissues were obtained at euthanasia, immediately fixed in formalin, and embedded in paraffin. Sections (4μM) were cut. IL-6 and amyloid A expression were evaluated in 7 baboon livers by immunohistochemistry (IHC) (13). After xylene, they were rehydrated, endogenous peroxidase activity was eliminated, and antigen was retrieved. The samples were incubated with the primary and secondary antibodies, followed by diaminobenzidine staining, and examined with an optical microscope (Olympus Optical, Tokyo, Japan). For staining, the primary antibodies were rabbit polyclonal IL-6 antibody (ab6672, Abcam, Cambridge, UK), and rat anti-amyloid A (Accuplex, Maynooth, Co Kildare, Ireland) and the secondary antibodies were goat anti-rat and goat anti-rabbit (Ab#97057, Cat#Ab6721, Abcam, Cambridge, MA). Quantitative analysis of expression was determined by measurement of the mean optical density (MOD) using Image J software (National Institute of Mental Health, Bethesda, MA). Five visual fields of each sample were randomly selected, and 5 areas were randomly selected from each visual field. The mean optical density was calculated finally.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 7 software (GraphPad Software, La Jolla, CA). Continuous variables were expressed as mean +/- SD. The unpaired Welch`s t test or Mann-Whitney U-test for variables (such as serum creatinine, WBC, platelet, fiboringen, SAA score, inflammatory factors). The Spearman rank correlation respectively analyzed correlations between serum creatinine and other variables, between SAA and inflammatory factors (such as baboon or pigIL-6), between serum baboon IL-6 and IL-6 or amyloid A in baboon livers. Differences were considered to be statistically significant at $p<0.05$.

RESULTS

Clinical Course

Based on the clinical course and graft histopathology, the baboons were divided into 2 groups - Rejection group (n=6) and Infection group (n=3). In the Rejection group, 5 baboons received conventional immunosuppressive therapy, and only one received an anti-CD40mAb-based regimen, whereas all 3 baboons in the Infection group received the anti-CD40 mAb-based regimen. In the Rejection group, one pig kidney underwent hyperacute rejection, and 5 underwent acute humoral xenograft rejection. In the Infection group, all 3 baboons were euthanized for systemic infection (at 136, 237, and 260 days) with functioning grafts (**Table 1**).

Serum Creatinine

An increase in serum creatinine was the most important indicator of failing pig kidney function after xenotransplantation (**Figure 1A**). Serum creatinine rose in the days before euthanasia became necessary (euthanasia vs pre-Tx, 1.96+/-0.45 vs 0.69+/-0.05mg/dL, $p<0.05$) (**Figure 1B**), but there was no significant difference between the Rejection and Infection groups on day 1 or at euthanasia (day 1, 1.13+/-0.31 vs 1.03+/-0.28mg/dl; at euthanasia, 2.12+/-0.50 vs 1.63+/-1.03mg/dL) (**Figure 1C**), although in the Infection group the serum creatinine was significantly increased in only one baboon (B9313) at euthanasia.

Systemic Inflammatory Response

A systemic inflammatory response after transplantation was detected by increases in SAA levels, especially at the time of euthanasia (**Figures 2A, B**). In the Rejection group, B14115 died from hyperacute rejection on day 1, when the SAA was significantly increased (**Figure 2A**). In the remaining 5 baboons, SAA levels were significantly increased in 3, and moderately

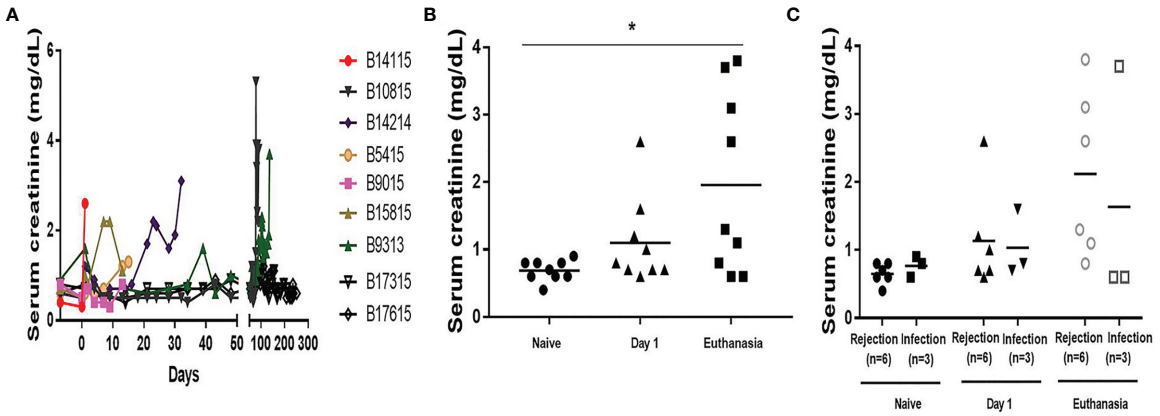

FIGURE 1 | Serum creatinine in baboons with pig kidney grafts. **(A)** Levels of serum creatinine in the baboons (n=9) at different time-points. **(B)** The difference of the levels of serum creatinine in the baboons (n=9) at pre-Tx (naïve), day 1 after operation, and at euthanasia. **(C)** The differences in serum creatinine between the rejection (n=6) and infection (n=3) groups at pre-Tx (naïve), day 1 after operation, and at euthanasia. (*p < 0.05).

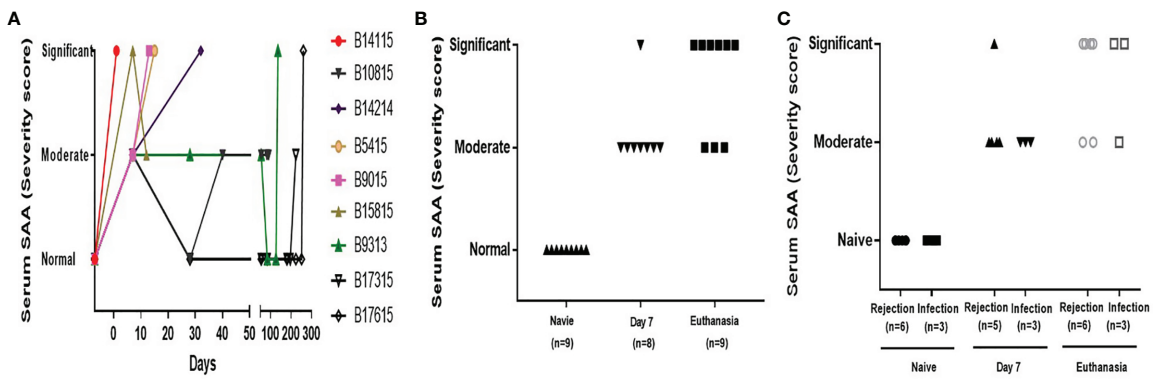

FIGURE 2 | Serum amyloid A in baboons with pig kidney grafts. **(A)** SAA scores in the baboons (n=9) at different time-points. **(B)** The difference in SAA scores in the baboons (n=9) at pre-Tx (naïve), day 7 after operation, and at euthanasia. **(C)** The difference in SAA scores between the rejection (n=6 or 5) and infection (n=3) groups at pre-Tx (naïve), day 7 after operation, and at euthanasia. The severity of the systemic inflammatory response was based on the results of the SAA assay, using a simple scoring system (1 = no inflammation; 2 = moderate inflammation; 3 = severe inflammation).

increased in 2 (**Figure 2C**). In the Infection group, SAA was increased during the first week and significantly or moderately increased at euthanasia (**Figure 2C**). In the absence of rejection or infection, the SAA remained negative or moderate.

White Blood Cell (WBC) and Platelet Counts and Plasma Fibrinogen

The WBC count was significantly decreased following induction therapy, and remained low (**Figure 3A**, left and middle). On post-transplant day 7, there was no statistical difference between the groups (3840+/-1185 vs 2400+/-379/μL) (**Figure 3A**, right). At euthanasia, the mean WBC count in the Infection group was higher than in the Rejection group, but with no significant difference (9233+/-4413 vs 5333+/-1595/μL) (**Figure 3A**, right).

Platelet counts remained within the normal range in both groups on day 1 (452+/-91 vs 315+/-33/μl, p>0.05) (**Figure 3B**, right). At euthanasia, the mean platelet count in the Rejection group was lower than in the Infection group (167+/-85 vs 356+/-41/μl), but was not significantly different (p=0.09) (**Figure 3B**, right).

Changes in plasma fibrinogen were similar to those in platelet count (**Figure 3C**, left and middle). It was maintained within the normal range at day 1 after operation in both groups (230+/-26 vs 192+/-24mg/dl, p>0.05) (**Figure 3C**, right). At euthanasia, it was lower in the Rejection group than in the Infection group (234+/-35 vs 124+/-28/μl) but was not significantly different (p=0.06), especially in 2 baboons (B9015, B15815) (**Figure 3C**, right).

Serum Cytokines

Serum *baboon* IL-6 was significantly increased on day 1 and at euthanasia (**Figure 4A**, left and middle). In the Rejection group, the levels of serum baboon IL-6 were increased on day 1, particularly in B14115 that died from hyperacute rejection

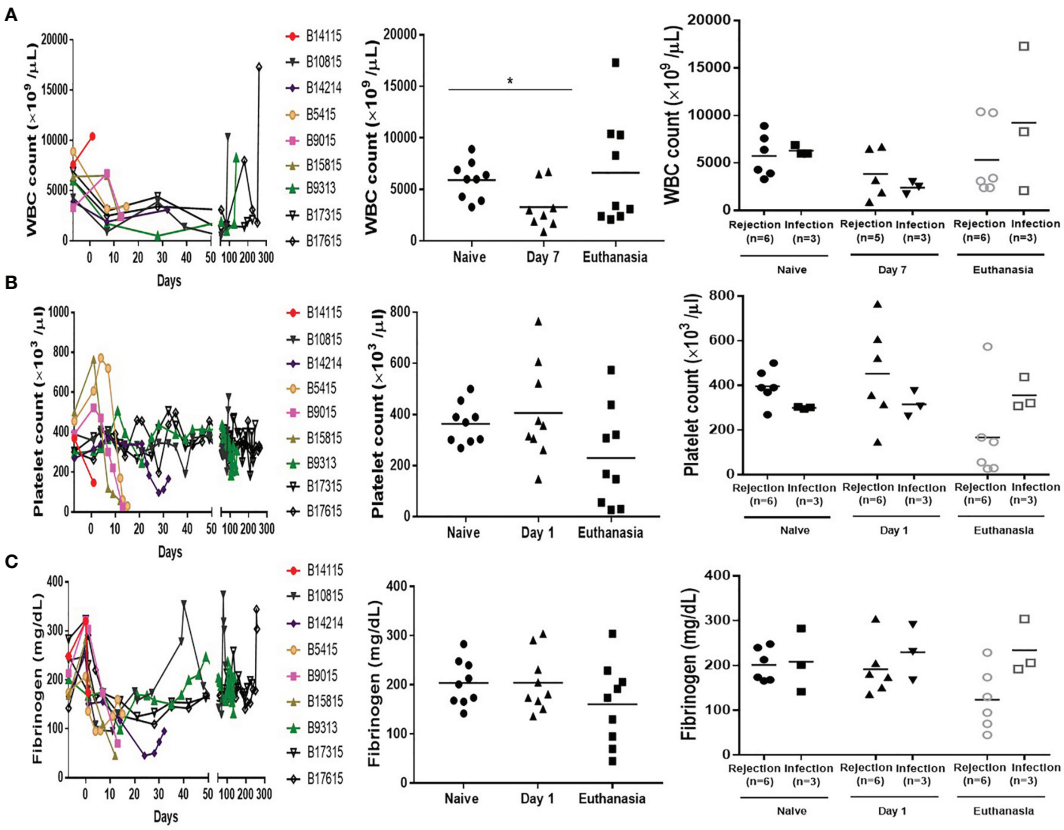

FIGURE 3 | White blood cell and platelet counts, and plasma fibrinogen levels in baboons with pig kidney grafts. **(A)** WBC counts in the baboons (n=9) at different time-points (Left); The difference in the WBC count in the baboons (n=9) (Middle), and between the rejection (n=6 or 5) and infection (n=3) groups at pre-Tx (naïve), day 7 after operation, and at euthanasia (Right). **(B)** Platelet counts in the baboons (n=9) at different time-points (Left); The difference in the platelet count in the baboons (n=9) (Middle), and between the rejection (n=6) and infection (n=3) groups at pre-Tx (naïve), day 1 after operation, and at euthanasia (Right). **(C)** Plasma fibrinogen in the baboons (n=9) at different time-points (Left); The difference in the plasma fibrinogen in the baboons (n=9) (Middle), and between the rejection (n=6) and infection (n=3) groups at pre-Tx (naïve), day 1 after operation, and at euthanasia (Right). (*p < 0.05).

(9260pg/mL); in the remaining 5 the levels of serum baboon IL-6 ranged from 516 to 3636pg/mL. The levels of serum *baboon* IL-6 were also significantly increased (range from 272 to 748pg/mL) in the Infection group, but were higher in the Rejection group (3291+/-1273 vs 456+/-148pg/mL, p<0.05) (**Figure 4A**, right). At euthanasia, the levels were high in both groups, and not statistically different Rejection group 4847+/-2769 vs Infection group 1943+/-1178pg/mL) (**Figure 4A**, right).

Serum *pig* IL-6, produced by the pig kidney graft, was detectable in some baboons in both groups on day 1 and at euthanasia, especially in B14115 (159pg/mL) and B9313 (125pg/mL) (**Figure 4B**, left and middle). Although higher in the Rejection group than in the Infection group on day 1 (44+/-25 vs 7+/-4pg/mL), and lower than in the Infection group at euthanasia (37+/-26 vs 42+/-42 pg/mL), there was no significant difference on day 1 or at euthanasia (**Figure 4B**, right).

Serum TNF-α was significantly elevated on day 1, and then recovered or was elevated at euthanasia in both groups (**Figure 4C**, left and middle). It was lower in the Rejection group than in the Infection group on day 1 (25+/-6 vs 46+/-5pg/mL, p<0.05). The levels of serum TNF-α in 4 baboons (B10815, B9313, B17315, B17615) with long survival were very high on day 1 (range 37-53pg/mL), but there was no difference between the two groups at euthanasia (10+/-3 vs 12+/-10pg/mL, p>0.05) (**Figure 4C**, right).

The levels of serum IL-1β were low throughout the course of the experiments (**Figure 4D**, left and middle), The detection rate and levels of serum IL-1β were higher in the Rejection group than in the Infection group on day 1, In only one baboon (B17615) was a low level of serum IL-1β detected in the Infection group. There was no statistica0y difference between the two groups at euthanasia (3+/-3 vs 1+/-1pg/mL, p >0.05) (**Figure 4D**, right).

Correlation of Serum Creatinine With SAA and Serum Cytokines

Significant positive correlations were found between serum creatinine and SAA (r=0.56, p<0.01) (**Figure 5A**). Serum

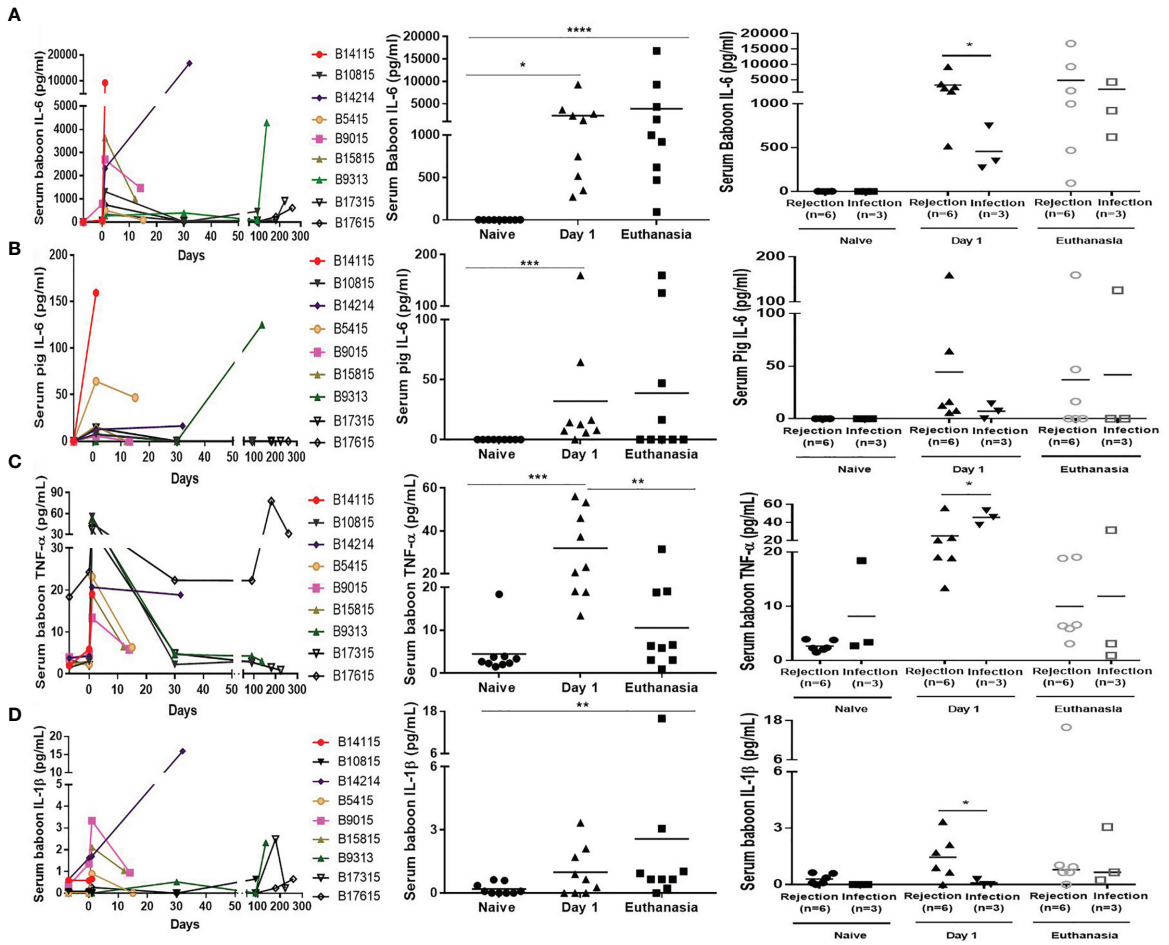

FIGURE 4 | Levels of serum cytokines in baboons with pig kidney graft. **(A)** Levels of serum baboon IL-6 in the baboons (n=9) at different time-points (Left); The difference of serum baboon IL-6 in the baboons (n=9) (Middle), and between the rejection (n=6) and infection (n=3) groups at pre-Tx (naïve), day 1 after operation, and at euthanasia (Right). **(B)** Levels of serum pig IL-6 in the baboons (n=9) at different time-points (Left); The difference of serum pig IL-6 in the baboons (n=9) (Middle), and between the rejection (n=6) and infection (n=3) groups at pre-Tx (naïve), day 1 after operation, and at euthanasia (Right). **(C)** Levels of serum TNF-α in the baboons (n=9) at different time-points (Left); The difference of serum TNF-α in the baboons (n=9) (Middle), and between the rejection (n=6) and infection (n=3) groups at pre-Tx (naïve), day 1 after operation, and at euthanasia (Right). **(D)** Levels of serum IL-1β in the baboons (n=9) at different time-points (Left); The difference of serum IL-1β in the baboons (n=9) (Middle), and between the rejection (n=6) and infection (n=3) groups at pre-Tx (naïve), day 1 after operation, and at euthanasia (Right). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

baboon IL-6, serum pig IL-6, and serum IL-1β (but *not* serum TNF-α) also positively correlated with serum creatinine (r=0.59, p<0.001; r=0.58, p<0.001; r=0.44, p<0.01, respectively) (**Figures 5E–H**), but serum creatinine did not correlate with WBC or platelet counts, or plasma fibrinogen (**Figures 5B–D**).

Correlation of SAA With Cytokines (Serum Baboon/Pig IL-6, Serum IL-1β)

SAA and serum cytokines (serum baboon/pig IL-6, serum IL-1β) positively correlated with serum creatinine. The correlation between the inflammatory response and cytokines was further be analyzed. Significant changes in serum baboon IL-6 correlated with SAA (r = 0.58, p< 0.01) (**Figure 6A**). However, the level and detection rate of serum pig IL-6 was lower than of serum baboon IL-6. Serum pig IL-6 also positively correlated with SAA (r = 0.61, p<0.001) (**Figure 6B**). IL-1β is mostly secreted by monocytes or macrophages, and is involved in inflammatory disease (25, 26). Correlation analysis showed that IL-1β positively correlated with SAA (r=0.41, p<0.05) (**Figure 6C**).

Serum Baboon IL-6 Level Is Associated With Expression of IL-6 or Amyloid A in the Baboon Livers

Expression of IL-6 and amyloid A in the native livers in (i) a baboon that experienced anaphylactic shock after ATG administration (without xenotransplantation or anti-inflammatory therapy) (B11015) (**Figure 7A**), and in (ii) a baboon that hyperacutely rejected a pig kidney graft (B14115)

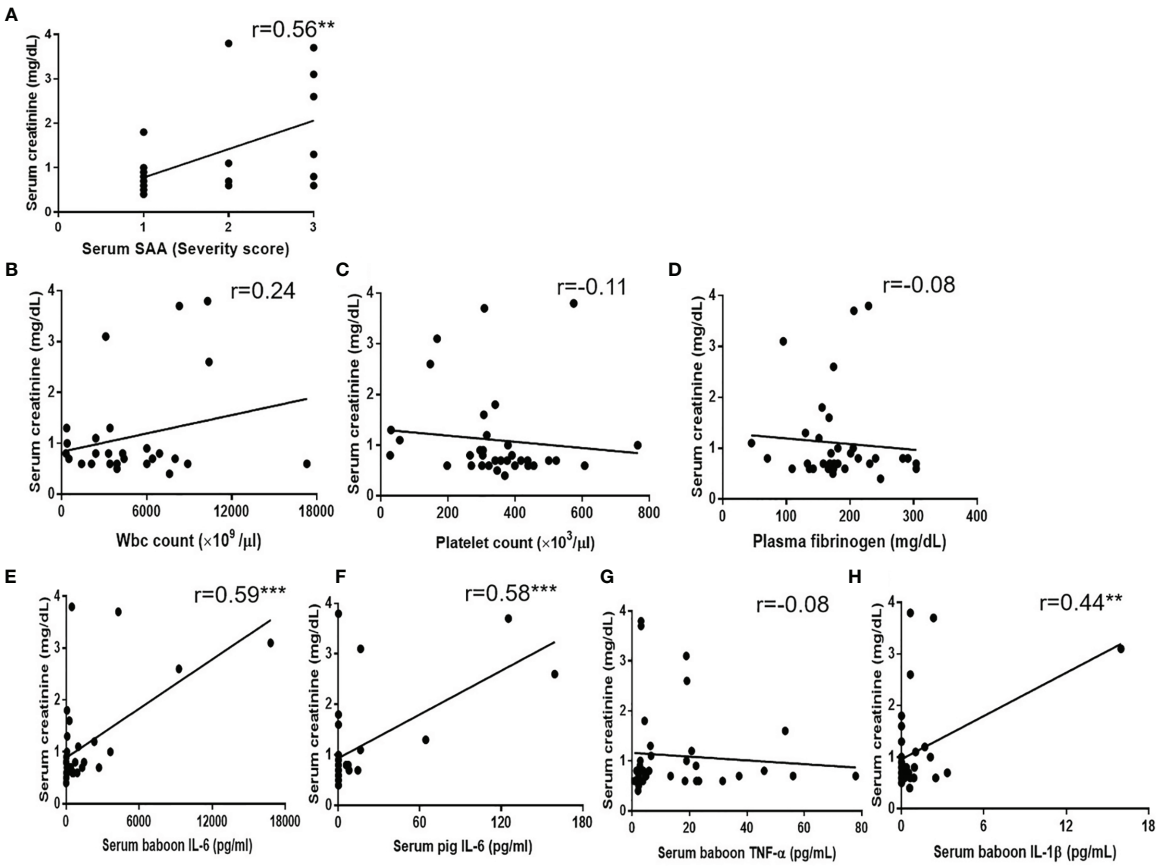

FIGURE 5 | Correlations between serum creatinine with **(A)** SAA score, **(B)** WBC count, **(C)** platelet count, **(D)** plasma fibrinogen, **(E)** serum baboon IL-6, **(F)** serum pig IL-6, **(G)** serum TNF-α, **(H)** serum IL-1β. Significant positive correlations were found between serum creatinine and SAA score (**p < 0.01), serum baboon IL-6 (***p < 0.001), serum pig IL-6 (***p < 0.001) or serum IL-1β (**p < 0.01).

(**Figure 7B**), showed highly-positive staining (and high serum levels of IL-6 and SAA), and were used as positive controls. IL-6 and amyloid A staining in baboon livers (B9015, B9313) represented the *Rejection* (**Figure 7C**) and Infection (**Figure 7D**) groups.

Expression of IL-6 and amyloid A were strongly positive in 7 baboon livers (Rejection group, n=6, and Infection group, n=1). Serum baboon IL-6 positively correlated with IL-6 expression in the baboon livers (r=0.93, p<0.01) (**Figure 7E**), and with amyloid A expression (r=0.79, p<0.05) (**Figure 7F**).

DISCUSSION

We previously documented evidence of a persistent systemic inflammatory response (SIXR) in immunosuppressed baboons after pig kidney xenotransplantation. SIXR was associated with procoagulant and inflammatory innate immune cells, and possibly with failure of the pig xenograft (3–7).

When a pig organ is exposed to SIXR, thrombocytopenia and a fall in fibrinogen indicate the development of thrombi in the pig graft (7, 23). In the present study, our data showed early dramatic falls in platelet count and plasma fibrinogen before euthanasia that preceded a rise in serum creatinine. This was most obvious in baboons receiving conventional immunosuppressive therapy. Therefore, we suggest that falls in platelet count or plasma fibrinogen may be precursors of graft failure induced by rejection, but are not associated with infection. However, falls in platelet count or plasma fibrinogen did not significantly correlate with the rise in serum creatinine.

Measurement of acute phase proteins, e.g., C-RP, SAA, is known to be valuable in monitoring for a systemic inflammatory response, and these can be promoted by IL-6 (8). IL-6R blockade, e.g., with tocilizumab, has been shown to be beneficial in allo- and possibly xeno-transplantation (27–30). In our previous studies in baboons with pig kidney grafts, treatment with tocilizumab completely prevented a rise in C-RP even when graft failure or SIXR was developing (4, 14). However, the level of SAA was not affected by tocilizumab, and

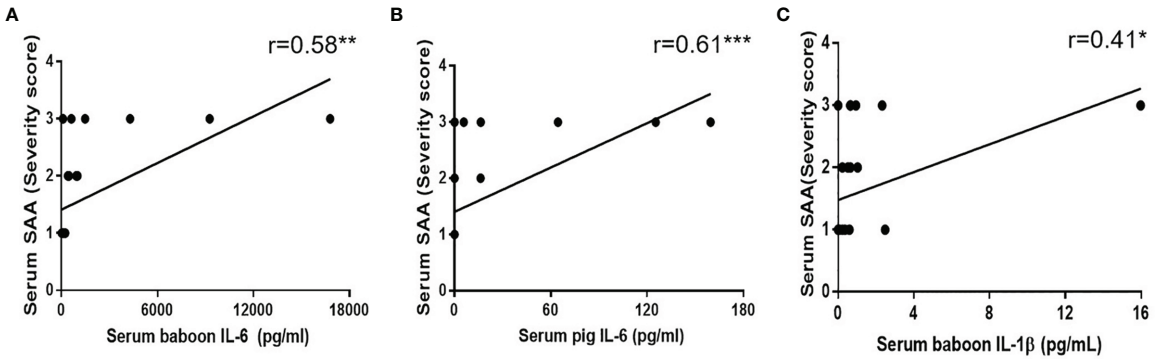

FIGURE 6 | Correlation between SAA score with **(A)** serum baboon IL-6, **(B)** serum pig IL-6, **(C)** serum IL-1β. Significant positive correlations were found between SAA score and serum baboon IL-6 (**p < 0.01), serum pig IL-6 (***p < 0.001) or serum IL-1β (*p < 0.05).

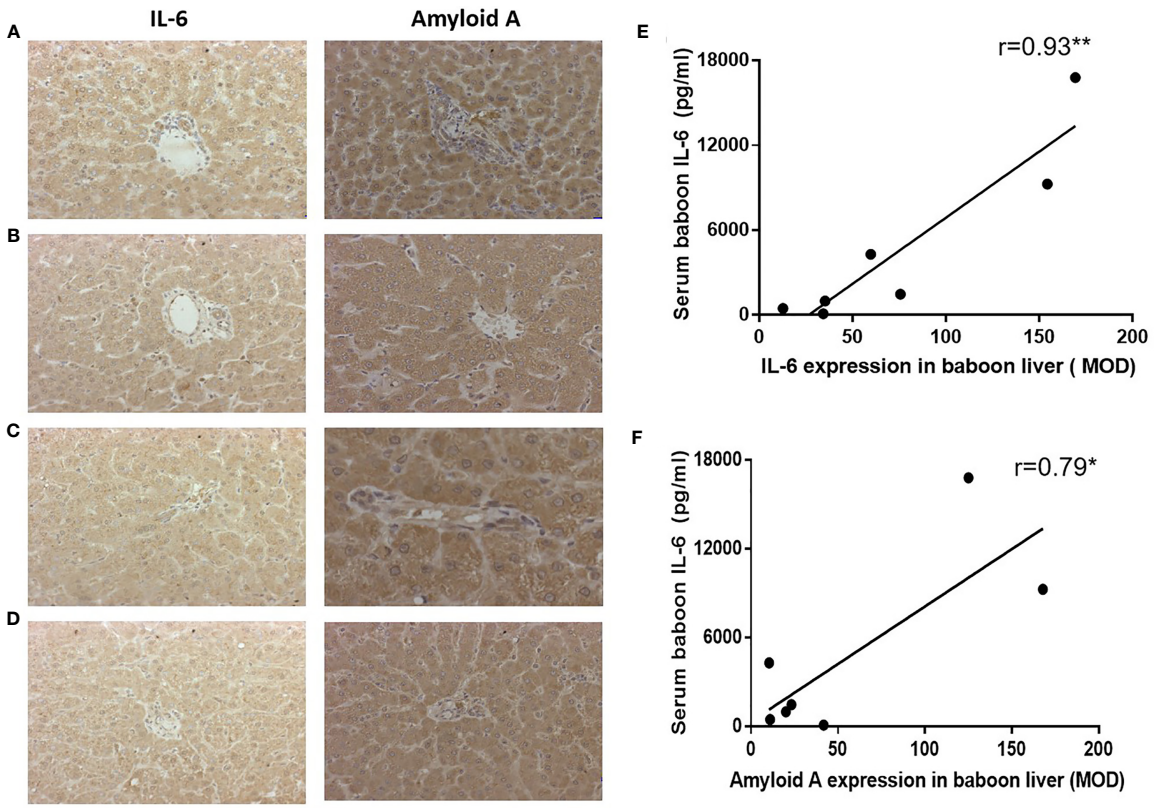

FIGURE 7 | Expression of IL-6 or amyloid A in baboon livers detected by IHC, and correlation with serum baboon IL-6 levels. **(A)** Expression of IL-6 and amyloid A in the liver of a baboon that experienced anaphylactic shock (without transplantation) (positive control), **(B)** Expression of IL-6 and amyloid A in the liver of a baboon with a pig kidney graft that underwent hyperacute rejection (while receiving tocilizumab and etanercept) (positive control). **(C)** Expression of IL-6 and amyloid A in the liver of a representative baboon with a pig kidney graft that was undergoing antibody-mediated rejection (while receiving tocilizumab and etanercept). **(D)** Expression of IL-6 and amyloid A in the liver of a representative baboon with a pig kidney graft that was undergoing serious infection (while receiving tocilizumab and etanercept). **(E, F)** Significant positive correlations were found between serum baboon IL-6 levels and expression of IL-6 (**p < 0.01) or amyloid A (*p < 0.05) in baboon livers.

remained significantly increased, and therefore appeared to be a more reliable indicator of SIXR (13). Our present study indicates that the rise in SAA was associated with a rise in serum creatinine, but monitoring of SAA could not distinguish between rejection or infection, as both were associated with a high level of SAA.

IL-6 is a pro-inflammatory cytokine produced by innate immune cells, including monocytes and macrophages, lymphocytes, endothelial cells, and dendritic cells after recognition of the danger signals (31, 32). In our previous studies, high levels of serum IL-6 were detected in baboon recipients of pig kidney grafts, and were associated with inflammation and coagulation dysregulation (7, 18). In the present study, the level of serum baboon IL-6 in baboons that were euthanized or died from rejection was significantly increased, and was higher than in those euthanized for infection. Serum baboon IL-6 correlated with increases in serum creatinine and SAA, and with expression of amyloid A and IL-6 in the baboon liver. Therefore, serum baboon IL-6 could be a useful indicator for inflammation and impending pig graft failure. Although low, the level of serum pig IL-6 was also positively associated with the increase in SAA and serum creatinine, and may be a supplementary indicator for inflammation and pig graft failure.

TNF-α is another pro-inflammatory cytokine that can regulate the expression of pro-inflammatory-related genes, tissue factor, and leukocyte antigen class I in porcine aortic endothelial cells, and may contribute to activation of complement and procoagulant changes in porcine endothelial cells, with increased tissue factor expression (16, 17, 33, 34). Strategies that block TNF-α may prove useful in xenotransplantation (35, 36). In the present study, a high level of TNF-α was observed on day 1, suggesting that the initial rise of TNF-α was related to the surgical procedure. Etanercept (an anti-TNF-α antibody), beneficial in the treatment of rheumatoid arthritis (37, 38), might be beneficial in baboons undergoing pig organ transplantation if administered on the day of operation or on the previous day. However, our data did not show that the level of TNF-α correlated with the serum creatinine.

Human IL-1β can induce the expression of adhesion molecule genes, chemokines, and tissue factor in pig aortic endothelial cells, and human IL-1β cooperates with TNF-α to induce expression of swine leukocyte antigen (SLA) class-I (16). Pig IL-1β can also activate human umbilical vein endothelial cells (17). It is suggested that IL-1β is likely to promote inflammation and coagulation after pig organ xenotransplantation. However, IL-1β levels did not increase after pig artery patch or heart graft xenotransplantation (39). In the present study, IL-1β was increased at the time of euthanasia, and appeared to be higher during rejection than infection. Changes in IL-1β correlated with those in serum creatinine and SAA, suggesting that IL-1β could be a supplementary indicator of inflammation and impending pig kidney graft failure.

In summary, the present study suggests a relationship between SIXR and pig graft failure. Measurement of serum creatinine, SAA, and pro-inflammatory cytokines (serum baboon and pig IL-6 and IL-1β) in baboon recipients may prove useful in indicating whether a baboon recipient is developing rejection or infection, though it might be insufficient to distinguish conclusively between the two. However, whether increases in pro-inflammatory factors (serum baboon and pig IL-6 and IL-1β) are simply markers of inflammation or are definitively contributing to pig kidney graft failure requires further investigation. Nevertheless, there was a correlation between SAA and serum creatinine. Similarly, serum baboon and pig IL-6 and IL-1β (especially serum IL-6) correlated with SAA and serum creatinine. Therefore, serum baboon and pig IL-6 and SAA may serve as useful biomarkers for inflammation and pig kidney graft failure associated with rejection or infection, and might allow improved monitoring and management of primates with pig organ grafts.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All procedures were approved by the Institutional Animal Care and Use Committees of the University of Pittsburgh and the University of Alabama at Birmingham.

AUTHOR CONTRIBUTIONS

GW and HH designed the research and supervised the experiment. GZ carried out the experiments, analyzed the data, and drafted the article. HI, QL, TY, and AJ contributed to the experiments. DA provided the pigs (from Revivicor, Blacksburg, VA). DC, ME, and HH revised the article. All authors contributed to the article and approved the submitted version.

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Liver and Hepatocyte Transplantation: What Can Pigs Contribute?

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About one-fifth of the population suffers from liver diseases in China, meaning that liver disorders are prominent causative factors relating to the Chinese mortality rate. For patients with end-stage liver diseases such as hepatocellular carcinoma or acute liver diseases with life-threatening liver dysfunction, allogeneic liver transplantation is the only life-saving treatment. Hepatocyte transplantation is a promising alternative for patients with acute liver failure or those considered high risk for major surgery, particularly for the bridge-to-transplant period. However, the lack of donors has become a serious global problem. The clinical application of porcine xenogeneic livers and hepatocytes remains a potential solution to alleviate the donor shortage. Pig grafts of xenotransplantation play roles in providing liver support in recipients, together with the occurrence of rejection, thrombocytopenia, and blood coagulation dysfunction. In this review, we present an overview of the development, potential therapeutic impact, and remaining barriers in the clinical application of pig liver and hepatocyte xenotransplantation to humans and non-human primates. Donor pigs with optimized genetic modification combinations and highly effective immunosuppressive regimens should be further explored to improve the outcomes of xenogeneic liver and hepatocyte transplantation.

Keywords: coagulation disorders, hepatocyte xenotransplantation, hyperacute rejection, liver xenotransplantation, thrombocytopenia

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INTRODUCTION

With the rise in affluence, China has experienced a surge in the prevalence of liver disease, and the population effects have significant implications for global health (1). It is estimated that over one-fifth of the population in China is affected by some form of liver disease, notably hepatitis B virus, liver cirrhosis, liver cancer, and non-alcoholic fatty liver disease, making liver diseases one of the most significant contributions to health problems of the populace (2). Allogeneic liver transplantation is a life-saving treatment for end-stage liver diseases, hepatocellular carcinoma, or acute liver disease with life-threatening liver dysfunction (3–5). Liver transplantation can restore patients' normal health and lifestyle and extend their lifespan by 15 years (6). According to the data

from the Scientific Registry of Transplant Recipients, the overall survival rate of patients reached 90% one year after the death of a donor and 77% within five years (7). At present, the severe imbalance between liver supply and demand has become the main bottleneck restricting the application of liver transplantation. In the past ten years, the number of liver transplants in the United States has increased by 31% (8). More than 51,000 patients have died while waiting for liver transplants in the last 20 years (9).

To address the issue of organ supply, the clinical application of porcine xenogeneic livers remains an attractive goal. In addition to the advantages of physiological indicators and fewer ethical issues (10–12), considerable knowledge and experience have been gained regarding tissue typing and genetic engineering, making pigs more likely to become optimal donors (13). At the same time, the porcine endogenous retrovirus (PERV), which is the core of the pathogen's cross-species infection, has also been knocked out in donor pigs, and the breeding of PERV knockout pigs has been realized (14, 15). The genetically modified donor pigs have achieved rapid, efficient, and diversified growth (16). The survival time of such pigs' organs in non-human primates (NHPs) has been continuously extended. The longest survival time of recipients after orthotopic renal transplantation was 499 days (17), while the survival time of ectopic and orthotopic heart transplantation has reached up to 945 days and 195 days, respectively (18, 19).

As acute liver failure caused by chemical or viral hepatitis often has a sudden onset, it is usually impossible to determine a suitable donor organ before severe coagulopathy and/or death occurs (10). Due to the risk of major surgery in patients with severe liver diseases, alternative methods are needed to support liver function and regenerate the injured liver for those waiting for liver transplantation (20). Human hepatocyte transplantation has been successfully used in patients with acute liver failure and metabolic liver diseases (21–26). Meanwhile, porcine hepatocyte xenotransplantation has shown significant improvement of clinical parameters in small animal models of acute or chronic liver failure (27, 28). Since porcine hepatocyte xenotransplantation is much less invasive and allows the use of encapsulated and/or genetically modified cells, it provides an innovative approach to temporarily supporting liver function (29).

This brief review will summarize the development and potential therapeutic impact of pig liver and hepatocyte xenotransplantation in humans and NHPs as well as the remaining barriers that currently prevent widespread clinical application, including rejection, thrombocytopenia, and blood coagulation dysfunction.

1 PIG LIVER XENOTRANSPLANTATION

1.1 Wild-Type Pig-to-Human and -NHP Liver Xenotransplantation

Makowa et al. performed auxiliary liver transplantation in 1993 for a grade 3–4 hepatic coma patient with fulminant liver failure caused by autoimmune hepatitis using a wild-type (genetically unmodified) pig liver (30). Pre-treatment of the patient *via* the plasma elution method and specific antibody removal before the operation eliminated 90% of the natural anti-pig antibodies in the circulating blood of the recipient. The liver xenograft functioned, as shown by bile production and stabilization of prothrombin levels, and the liver function indicators tended to expected values after transplantation. In the end, the donor liver survived for 20 hours, and the recipient survived for 34 hours, demonstrating the ability of a pig liver to function at least temporarily. The autopsy revealed antibody-mediated immune rejection in the liver, suggesting that the heterogeneous antibodies in the circulating blood of the recipient could increase rapidly after being cleared. After the initial clinical failure of xenotransplantation studies using pigs as donors, the focus shifted to the field of basic research to find a way to eliminate rejection.

The first problem faced when a wild-type pig organ is transplanted into a patient or an NHP is the hyperacute rejection (HAR) associated with the binding of primate preformed antibodies to pig antigens. HAR leads to intravascular thrombosis, hemorrhagic necrosis, endothelial cell injury, and deposition of IgM, IgG, and C3 (10). For example, the first study in experimental unmodified pig-to-NHP liver xenotransplantation (LXT) was performed by Calne et al. in 1968 (31). Four of the seven baboons died of uncontrollable bleeding 6–30 hours after surgery. The longest survival time for the remaining recipients was only 3.5 days. Pathological examination showed that a large number of immune-inflammatory cells infiltrated the portal area. Subsequently, researchers used rhesus monkeys and gorillas as recipients; the animals survived less than 12 hours. During the next 20 years, the recipients' survival time never exceeded three days, and thus the LXT studies at this stage all failed with wild-type pigs as donors. Therefore, genetically modified pigs were produced with the intention of alleviating rejection.

1.2 Genetically Modified Pig-to-NHP Liver Xenotransplantation

1.2.1 Orthotopic Liver Xenotransplantation

The incidence of HAR was lowered when using organs from pigs expressing one or more human complement regulatory proteins (hCRPs) such as CD46, CD55, or CD59 (32) as the hCRPs play roles in repressing the recipients' complement activation. In 2000, Ramirez et al. implemented the first orthotopic xenogeneic liver transplantation in baboons using genetically modified pigs expressing CD55 as donors (33). Neither liver xenograft demonstrated histopathological features of HAR. The baboons survived up to eight days. After that, medical researchers used livers from transgenic pigs with CD55, CD59, and H-transferase (34). Albeit the transplanted livers did not show obvious HAR and maintained a complete liver structure, the recipients experienced thrombosis, and survival time was only 13–24 h.

For HAR, the main target antigen is galactose-α-1,3-galactose, an oligosaccharide not present in humans, apes, or Old World monkeys (35, 36). Cooper et al. and Ekser et al. used

α-1,3-galactosyltransferase gene-knockout (GTKO) minipigs expressing CD46(GTKO/hCD46) as donors to perform LXT (37, 38). The transplanted liver showed normal liver function according to relevant indicators in peripheral blood after the operation. However, the recipients experienced severe thrombocytopenia, leading to eventual death from abdominal bleeding and limiting recipient survival to a maximum of seven days. For severe thrombocytopenia after LXT, Kim et al. performed postoperative Amicar treatment of the recipients to inhibit fibrinolysis and prolonged the survival time of the recipients to nine days (39). Although the recipients did not develop fatal thrombocytopenia, they still suffered severe blood loss and sepsis. Navarro-Alvarez et al. supplemented human coagulation factors to recipients; however, these factors had no positive effect on prolonging survival (40). Baboons quickly developed extensive vessel thrombosis and thrombotic microangiopathy. Persistent thrombocytopenia, porcine graft thrombotic microangiopathy (TMA), and consumptive coagulopathy have been considered to be due to interspecies incompatibilities between pigs and NHPs (40).

Shah et al. documented 25 and 29-day survival of pCMV-free GTKO pigs to baboons treated with administration of exogenous human coagulation factors along with costimulation blockade (41, 42). Immunosuppressive therapy of recipients included induction with anti-thymocyte globulin, KF-506, methylprednisolone, and costimulation blockade (belatacept and anti-CD40 mAb, respectively). Thrombocytopenia spontaneously recovered within a few days after surgery without the need for platelet transfusions to recipients. The autopsies showed that the baboons had no evidence of rejection, inflammation, or TMA. These results recorded the longest survival time of pig-to-NHP LXT to date and represented an advance towards realistic consideration of the clinical applicability of LXT as a bridge to allotransplantation for patients.

1.2.2 Heterotopic Liver Xenotransplantation

In 2013, Dou's group transplanted a part of the liver from GTKO pigs as an auxiliary graft into Tibetan monkeys. Although the recipients required native splenectomy, none of the native liver needed to be excised, as the graft fitted comfortably into the splenic fossa. In theory, this can help control postoperative immune rejection and promote the recovery of the recipient's liver function and help maintain normal coagulation and anti-coagulation functions (43, 44). The grafted liver functioned, as manifested by the regular physiological and biochemical indicators, common coagulation system, and stable platelet numbers. Monkeys showed no HAR or severe acute rejection after the operation, and survival time reached up to 14 days.

Yeh et al. at Massachusetts General Hospital of the United States also conducted a heterotopic auxiliary liver transplantation trial. After the operation, the recipients were supplemented with exogenous full-spectrum coagulation factors. The transplanted liver and the recipient survived for 15 days, and the recipient eventually died of liver failure and infection (45).

The most recent report documented a 26-day survival of a 13-gene modified pig liver graft in a rhesus monkey in 2021. The specific genotype of the donor pig is *PERV*-KO/*GalT*-KO/*β4GalNT2*-KO/*CMAH*-KO/*hCD46*/*hCD55*/*hCD59*/*hβ2M*/*hHLA-E*/*hCD47*/*hTHBD*/*hTFPI*/*hCD39* (*PERV*-KO/3-KO/9-TG), corresponding to the most extensively genetically modified and humanized type of donor pig to date (15). The autopsy found that humoral rejection occurred in the transplanted liver accompanied by inflammatory damage, interstitial hemorrhage, and TMA, suggesting that the incorporation of nine human genes from the donor pig did not completely avoid xenograft damage. To a certain extent, this report provided encouragement in that *PERV*-KO/3-KO/9-TG pigs had advantages in inhibiting xenograft rejection and recipients' coagulation abnormalities.

2 PIG HEPATOCYTE XENOTRANSPLANTATION

Human hepatocyte transplantation could bridge patients with acute liver injury to liver transplantation until donor livers are available (46), which could even sometimes restore hepatic function. It is noted that significant barriers hinder broader implementation of human hepatocyte transplantation at present, such as limited supply of donor hepatocytes (25), engraftment and repopulation efficiency, cell viability (47), and allogeneic rejection (48). Hepatocytes from pigs are an alternative source that could be unlimited. Besides, transplanted porcine hepatocytes have no more immunogenic than their human counterparts and even could provide protection against human humoral and cellular immune responses (49, 50). There have been preclinical studies on xenotransplantation of hepatocytes from wild-type pigs and genetically-modified pigs into NHPs.

2.1 Wild-Type Pig-to-NHP Hepatocyte Xenotransplantation

The first study in experimental preclinical pig-to-NHP hepatocyte xenotransplantation was performed by Nagata et al. in 2007 using hepatocytes from wild-type pigs (51). According to the preliminary clinical experience of fetal hepatocyte allotransplantation, xenogeneic hepatocytes were transplanted into the spleen in three cynomolgus monkeys with normal liver functions, and regular immunosuppressive was used to control the rejection (52). The xenogeneic hepatocytes functioned for more than 80 days, and this was extended to 253 days following re-transplantation. In this study, xenogeneic hepatocyte transplantation did not appear to be affected by the anti-pig antibodies because the vascular endothelium of the liver was not transplanted (53, 54). The isolated xenogeneic hepatocyte transplantation lacks xenogeneic endothelial cells, and the latter are the most vulnerable to humoral immune damage. In this report, because the recipients had a normal liver function at the time of transplantation, it was impossible to predict the extent to which xenogeneic hepatocytes transplantation could restore human liver function.

Porcine hepatocytes microencapsulated in alginatepoly (L-lysine)-alginate microspheres and transplanted intraperitoneally in baboons with fulminant liver failure (FLF) demonstrated effectiveness (20). Capsules or microspheres are designed to protect cells against circulating antibodies and immune or inflammatory cells while allowing nutrients such as oxygen and glucose to diffuse inside the microspheres (55–57). Three of four baboons recovered completely with normal liver function. The remaining baboon developed liver failure but survived 21 days (much longer than the control group). Microencapsulated porcine hepatocytes provided temporary liver function in NHP in the first few days of FLF and increased survival rate, thereby opening new prospects for xenogeneic hepatocyte transplantation.

2.2 Genetically Modified Pig-to-NHP Hepatocyte Xenotransplantation

Iwase et al. (50) chose the spleen as the primary site for xenotransplantation in three baboons using hepatocytes from GTKO/hCD46 pigs. And other injection sites were selected according to previous reports in rodents, including lymph nodes (58–61), the subcapsular space of the kidney (62), and subcutaneous fat on the abdominal wall (63). The results were significantly different from Nataga's report (51), as porcine hepatocytes or infiltrating immune cells were not found in all recipients at any injection sites. The procedures to be optimized in further studies as follows: detect functional viability of the porcine hepatocytes, select a single injection site in one xenotransplantation, measure the cytokine and chemokine responses to hepatocytes, and use the porcine hepatocytes expressing human CD47 and/or HLA-E/G. In conclusion, more basic research is needed before xenogeneic porcine hepatocyte transplantation enters the clinic.

HAR is the most severe rejection when pig livers transplanted into NHPs. Other aspects include severe blood coagulation dysfunction, inflammation and spontaneous internal hemorrhage (32). HAR has been successfully overcome by using GTKO pigs. To mitigate the immunological and physiological molecular incompatibilities between the porcine graft and the human immune system, genetic engineered pigs should be exploited by knocking out more porcine-specific glycan epitopes, overexpression of the hCRPs and coagulation regulatory proteins alone or in combination (15).

3 DISCUSSION

In recent decades, basic research of pig-to-NHP LXT has developed rapidly. In the beginning, the leading cause of death of the recipients after LXT was HAR due to incompatibility between species; this led to the longest survival time being only nine days. With the emergence of GTKO and/or hCRPs pig donors, HAR has been basically controlled. The transplanted liver can produce relevant human proteins and maintain several indexes within the regular physiological ranges. However, the subsequent fatal coagulation characterized by severe thrombocytopenia, bleeding, and TMA has become the major obstacle (40).

Due to the use of aprotinin with the effect of inhibiting fibrinolysis, the survival period has been prolonged, and fatal thrombocytopenia no longer occurs in recipients, but blood loss persists. Continuous infusion of coagulation factors can ameliorate the thrombocytopenia and prevent TMA after LXT. The specific mechanism has yet to be elucidated and warrants further investigation (40). Supplementation of exogenous coagulation factors and costimulation blockade after the operation can not only effectively prolong the survival time of recipients but also prevent thrombocytopenia, coagulation disorders, and TMA (41, 42). The potential role of costimulation blockade in prolonging xenograft survival has been verified in other pig organ xenotransplantation models (64, 65).

Heterotopic auxiliary liver transplantation requires native splenectomy, and thus there is no need to excise the native liver; this can not only control postoperative immune rejection and promote the recovery of recipient liver function but also help maintain normal coagulation and anti-coagulation functions in recipients compared to orthotopic LXT (43, 44). The donor liver from the most extensively modified pigs (PERV-KO/3-KO/9-TG) still presents humoral rejection, inflammatory damage, interstitial hemorrhage, and TMA when transplanted into NHPs. These effects suggest that the expression of nine human genes from the donor pig does not entirely avoid xenograft damage by heterotopic auxiliary liver transplantation.

The genetic engineering of pigs renders their cells less immunogenic than their human counterparts (49, 66). The advantages of xenogenetic hepatocyte transplantation over whole liver transplantation are: i) no need to replace all liver functions because the recipient's own liver will not be removed; ii) being less invasive; iii) allowing the use of encapsulated and/or genetically modified cells; iv) less intense humoral immune response (29, 67), v) low morbidity and higher safety (5); vi) possibility of repeated administration. In contrast to whole organs, which are perfused through the blood vessels of the donor, cellular grafts derive their blood supply by the ingrowth of blood vessels of the recipient, a factor that may be the key to the success of cellular xenografts (68, 69).

Although the remaining issues hinder clinical implementation, donor pigs with optimized genetic modification combinations and highly effective immunosuppressive regimens should be further explored to improve the outcomes of xenogeneic liver and hepatocyte transplantation.

AUTHOR CONTRIBUTIONS

XL and HY contributed to select the topic of the manuscript. YW collected relevant references. XL wrote the first draft of the manuscript. HY, YD, and YW wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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Screening and Identification of the First Non-CRISPR/Cas9-Treated Chinese Miniature Pig With Defective Porcine Endogenous Retrovirus *pol* Genes

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Pig to human xenotransplantation is considered to be a possible approach to alleviate the shortage of human allografts. Porcine endogenous retrovirus (PERV) is the most significant pathogen in xenotransplantation. We screened for pigs that consistently did not transmit human-tropic replication competent PERVs (HTRC PERVs), namely, non-transmitting pigs. Then, we conducted whole-genome resequencing and full-length transcriptome sequencing to further investigate the sequence characteristics of one non-transmitting pig. Using *in vitro* transmission assays, we found 5 (out of 105) pigs of the Chinese Wuzhishan minipig inbred line that did not transmit PERV to human cells, i.e., non-transmitting pigs. Whole-genome resequencing and full-length transcriptome sequencing of one non-transmitting pig showed that all of the *pol* genes were defective at both the genome and transcript levels. We speculate that the defective PERV *pol* genes in this pig might be attributable to the long-term inbreeding process. This discovery is promising for the development of a strain of highly homozygous and genetically stable pigs with defective PERV *pol* genes as a source animal species for xenotransplantation.

Keywords: porcine endogenous retrovirus, Chinese miniature pig, defective gene, inbreeding, whole-genome resequencing, full-length transcriptome sequencing

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INTRODUCTION

Pigs are considered to be the most suitable donor animals for xenotransplantation because of their anatomical and physiological similarities to humans, large litter size, short gestation period and genetic malleability (1). However, the risk of transmission of porcine microorganisms to human xenotransplant recipients is a great concern. Many exogenous microorganisms can be eliminated from

donor herds by using various barrier methods and specific pathogen-free (SPF) breeding. However, porcine endogenous retroviruses (PERVs) cannot be eliminated in this way since they are integrated into the genomes of all pigs (2). PERVs can infect human cells *in vitro* under certain experimental conditions (3). Transspecies transmission has been shown for many retroviruses, including human immunodeficiency virus type 1 and 2 (HIV-1/2), human T-cell lymphotropic virus type 1 and 2 (HTLV-1/2), Koala retrovirus (KoRV) and Gibbon ape leukemia virus (GALV), and these viruses can cause more serious disease in newly infected hosts than in their natural hosts (4–8). Therefore, although transmission of PERVs has not been observed when animals (including nonhuman primates) were inoculated with PERV preparations, during preclinical xenotransplantation or in clinical transplantation to humans (9, 10), PERVs share the pathogenic potential and features common to other retroviruses and thus remain a major potential zoonotic risk in xenotransplantation.

PERVs are present in multiple copies (3 to 140 copies) in the pig genome (11). Three subtypes of PERVs were identified. PERV-A and PERV-B are present in the genome of all pigs and infect human cells as well, and PERV-C infects only pig cells and is not present in all pigs (12). In addition, recombinants of human-tropic PERV-A and ecotropic PERV-C have been detected, and one of the recombinant PERV-A/C viruses was found to be 500-fold more infectious than the prototype PERV-A (13). PERVs contain three open reading frames (ORFs), encoding *gag*, *pol* and *env*, that are located between two long terminal repeats (LTRs). The *pol* gene, encoding the reverse transcriptase, is highly conserved and is essential for PERVs' function.

In recent years, various strategies have been proposed to reduce the risk of PERV transmission to human recipients (14), including the selection of pig strains with low expression of PERV-A and PERV-B, the selection of pigs lacking PERV-C in the genome (in order to prevent recombination between PERV-A and PERV-C), the induction of neutralizing antibodies (15–17), the generation of transgenic pigs expressing PERV-specific small interfering (si)RNA (18–21) and gene editing using either a zinc finger nuclease (22) or clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology (23, 24) to inactivate all provirus copies in the genome. Among these, CRISPR-Cas9 technology has been the most promising approach to address the PERV problem. In 2015, Yang et al. (23), using CRISPR-Cas9 technology, succeeded in inactivating 62 PERV sequences in an immortalized pig cell line. In 2017, the same group reported the successful generation of PERV-inactivated pigs by somatic cell nuclear transfer (SCNT) from primary pig fetal fibroblast cells in which PERVs have been inactivated genome-wide using the CRISPR-Cas9 system (24). This approach may minimize the risk of PERV transmission after xenotransplantation and could provide donor animals suitable for xenotransplantation.

In this study, we reported the screening and identification of the first non-CRISPR/Cas9-treated Chinese miniature pig with defective PERV *pol* genes. We screened for pigs that consistently did not transmit human-tropic replication competent PERVs (HTRC PERVs), namely, non-transmitting pigs. Then, we conducted whole-genome resequencing and full-length transcriptome sequencing to further investigate the sequence characteristics of one non-transmitting pig. The results indicated that all the PERV *pol* genes in this pig's genome, as well as all the PERV *pol* transcripts, were defective.

MATERIAL AND METHODS

Animals

The pigs studied in this study were from a highly inbred strain of Chinese Wuzhishan minipigs (WZSPs), which was developed by the Institute of Animal Science of the Chinese Academy of Agriculture Science (CAAS) based on the inbreeding of one male and one female WZSP by full-sib mating over more than 24 generations. A total of 105 WZSPs were screened. Animal experiments were performed after receiving approval from the Beijing Experimental Animal Administration Committee (Beijing-SCXK-2015-0006) and according to the animal ethics guidelines.

Cell Lines

The cell lines used in these studies were obtained from the American Type Culture Collection (ATCC): human embryonic kidney cell line (HEK-293 cells, ATCC CRL-1573), porcine kidney cell line (PK-15 cells, ATCC CCL-33). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, USA) supplemented with 5% fetal bovine serum (Zhejiang Tianhang Biological Technology Co., Ltd., Hangzhou, China). Cells were maintained by subpassaging 1 or 2 times/week as needed.

PBMC Isolation and Activation

Blood (6 ml) was collected in ethylenediamine tetra-acetic acid tubes from each WZSP maintained by Grand Life Science and Technology Ltd., Beijing, China. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation in lymphocyte separation medium (Tianjin HAO YANG Biological Manufacture Co., Ltd., Tianjin, China). After three washes in phosphate-buffered saline (PBS), half of the PBMCs were cryopreserved in RPMI 1640 culture medium (Gibco, Carlsbad, CA, USA) supplemented with 40% fetal bovine serum and 10% dimethyl sulfoxide. The rest of the PBMCs were mitogenically stimulated in the following medium: RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 2.5 μg of phytohemagglutinin (PHA) per ml, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco, Carlsbad, CA, USA).

In Vitro Coculture Assay

Briefly, HEK293 cells were plated in standard medium in a six-well plate at a density of 3×10^5 cells per well. On the following day, approximately 6×10^5 PHA-stimulated PBMCs isolated from the blood of WZSPs were plated onto HEK293 cells. The PBMCs were kept in contact with HEK293 cells for 4 days. After that, the culture medium and PBMCs were removed and replaced with fresh medium, and the total RNA of HEK293 cells was harvested and amplified *via* RT-PCR to detect PERV elements in the

human cells. Then, the HEK293 cells cocultured with PBMCs derived from non-transmitting WZSPs were subcultured for a longer time, and HEK293 cells cocultured with PBMCs derived from a transmitting WZSP were cultured as a positive control. During this period, as HEK293 cells reached confluence, culture medium was collected for reverse transcriptase (RT) assays, and 10^6 cells were collected for genomic DNA and RNA isolation and subsequent PCR and RT-PCR test.

PCR and RT-PCR

Genomic DNA and total RNA were extracted from HEK293 cells with TRI Reagent (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) according to the manufacturers' instructions. Genomic DNA was used as a template for detecting pig GGTA1 to control for potential porcine genome contamination of HEK293 cells after coculture. Three microliters of DNA was added to a reaction mixture (in a final volume of 25 μl) containing 12.5 μl Premix Taq (Takara Biomedical Technology (Beijing) Co., Ltd., Dalian, China) and 1 pmol of each GGTA1 primer. Amplification was performed on a Veriti 96-well Thermal Cycler (Applied Biosystems, California, USA) under the following conditions: 1 cycle of 94°C for 5 min; 35 cycles of 94°C for 30 s, 56°C for 50 s, and 72°C for 45 s; and a final elongation step at 72°C for 5 min. RNA was converted to cDNA with M-MuLV reverse transcriptase (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocols. Genomic DNA and cDNA of HEK293 cells were then tested by PCR to detect PERV elements (*gag*, *pol* and *env*) in the human cells using three primer pairs as described previously (25). The cDNA of PK15 cells was used as a positive control. All the amplified products were evaluated by 1.5% agarose gel electrophoresis with ethidium bromide staining.

Reverse Transcriptase Activity Assay

RT activity in the culture supernatants was assayed by using an HS-Mn RT Activity Kit (Cavidi AB, Uppsala, Sweden) according to the manufacturer's protocol.

Whole-Genome Resequencing and Sequence Analyses

Genomic DNA was extracted from PBMCs of WZSP452 using a Wizard® genomic DNA purification kit (Promega (Beijing) Biotech Co., Ltd., Beijing, China) and then submitted to iGeneTech Bioscience Co., Ltd. (Beijing, China) for whole-genome resequencing on an Illumina HiSeq 4000 system (Illumina Inc., San Diego, CA, USA). Library preparation and sequencing as well as sequence data processing were conducted as previously described (26). After quality filtering, the remaining reads were mapped to the genomic data from the *Sus scrofa* breed Wuzhishan isolate L1-53 (GenBank accession number: AJKK01000000.1) using Burrows-Wheeler Alignment (BWA) (27). Then, the sequence of full-length proviral DNA of PERV-WZSP (GenBank accession number: EF133960.1), which had been previously derived from WZSP and sequenced by our group (28), was used as the reference sequence of PERV for the following sequence analysis. The pig genomes of WZSP452, Wuzhishan isolate L1-53 (GenBank accession number: AJKK01000000.1) and Duroc isolate TJ Tabasco (GenBank accession number: AEMK00000000.2) were screened with the *pol* gene of the PERV reference sequence by using SAMtools-0.1.19 (29). Then, the complete genome of the PERV-WZSP sequence was mapped onto the WZSP452 genomes with BLAST to detect full-length PERV insertions. The characteristics of PERV protein genes integrated into the WZSP452 genome were analyzed using the DNA Star software package (30).

Validation of Assembly *pol* Sequences in Scaffold640 and Scaffold5028 of WZSP452

The *pol* sequence in Scaffold640 contains 426 gaps (indicated by N). We designed the primers Scaffold640-polF (5'-AAGGGAAACAAAGGACTGAAGG-3') and Scaffold640-polR (5'-GAGTTCAGGCTGTCTCCTATGC-3') to close the gaps in Scaffold640 based on their flanking regions, and the fragment length amplified by this pair of primers is 3400 bp. Genomic DNA (6 μl) extracted from PBMCs of WZSP452 was added to a reaction mixture (in a final volume of 50 μl) containing 25 μl Q5® Hot Start High-Fidelity 2× Master Mix (New England Biolabs, Ipswich, MA, USA) and 5 pmol of each Scaffold640-*pol* primer. Amplification was performed on a Veriti 96-well Thermal Cycler (Applied Biosystems, California, USA) under the following conditions: 1 cycle of 94°C for 30 s; 35 cycles of 94°C for 10 s, 57°C for 30 s, and 65°C for 100 s; and a final elongation step at 65°C for 10 min. After treatment with Premix Ex Taq (Takara Biomedical Technology (Beijing) Co., Ltd., Dalian, China), the resultant deoxyadenosine triphosphate (dA)-tailed PCR products were characterized by 1% agarose gel electrophoresis and extracted using a TIANgel Midi Purification Kit (TIANGEN Biotech (Beijing) Co., Ltd., Beijing, China). Purified products were ligated into the pMD18-T vector (Takara Biomedical Technology (Beijing) Co., Ltd., Dalian, China) according to the manufacturer's instructions and transformed into *E. coli* DH5α competent cells (Takara Biomedical Technology (Beijing) Co., Ltd., Dalian, China). Plasmids were purified using the PureYield™ Plasmid Miniprep System (Promega (Beijing) Biotech Co., Ltd., Beijing, China). The insert size was confirmed by restriction digestion and analysis (*Sal*I and *Xba*I) (New England Biolabs, Ipswich, MA, USA). The plasmids containing inserts of the correct size were submitted to Beijing Biomed company (Beijing, China) for Sanger sequencing. Then, the sequences were aligned with the sequence of Scaffold640 using the DNA Star software package (30).

With regard to Scaffold5028, based on flanking regions of the premature termination codon of the PERV-*pol* gene, PCR primers Scaffold5028-*pol*F (5'-ACTTGGGAGTGGGACGGGTAAC-3') and Scaffold5028-*pol*R (5'-AATCCATCCCTGCGGTTTCTAC-3') were designed to amplify a 276 bp product. Genomic DNA (2 μl) extracted from PBMCs of WZSP452 was added to a reaction mixture (in a final volume of 50 μl) containing 25 μl Q5® Hot Start High-Fidelity 2× Master Mix (New England Biolabs, Ipswich, MA, USA) and 2 pmol of each Scaffold5028-*pol* primer. Amplification was performed on a Veriti 96-well Thermal Cycler (Applied Biosystems, California, USA) under the following conditions: 1 cycle of 98°C for 30 s; 35 cycles of 98°C for 10 s, 64°C for 30 s, and 72°C for 100 s; and a final elongation step at 72°C for 2 min. The resultant PCR products were submitted to Allwegene Tech. (Beijing, China) for high-throughput next-generation sequencing on the Illumina MiSeq platform (PE300).

Full-Length Transcriptome Sequencing

Transcriptome sequencing was performed by using single-molecule real-time (SMRT) sequencing, developed by Pacific Biosciences (PacBio). Briefly, total RNA was extracted from PBMCs derived from WZSP452 using TRI Reagent (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) according to the manufacturers' protocol. Then, according to the Isoform Sequencing protocol (Iso-Seq), Iso-Seq libraries were prepared using the SMARTer PCR cDNA Synthesis Kit (Clontech, Mountain View, CA, USA) and the BluePippin™ Size Selection System (Sage Science, Beverly, MA, USA) as described by Pacific Biosciences (Part Number 100-092-800-03). Sequencing was carried out on the Pacific Bioscience RS II platform (Pacific Biosciences, Menlo Park, CA, USA).

The raw data obtained by sequencing were processed using SMRTlink 5.0 software. Circular consensus sequences (CCSs) were generated from subread BAM files and then classified as full-length or non-full-length reads using 'pbclassify.py'. Reads without poly(A) tail signals and short reads (minimum sequence length = 200) were discarded. The full-length reads were then fed into the isoform-level clustering, namely, iterative clustering for error correction (ICE), followed by polishing using the Arrow function. Additional nucleotide errors in consensus reads were corrected using the Illumina RNA-seq data with the software LoRDEC (31). The consensus reads were aligned with GMAP (32) to the pig genome sequence and to the genome of the PERV-WZSP isolate (GenBank accession number: EF133960.1).

RESULTS

In Vitro Transmission Assays for Screening WZSPs With Non-Transmitting PERV

We conducted *in vitro* transmission assays to screen for WZSPs that did not transmit PERVs from pig to human cells. After coculture with PHA-stimulated PBMCs derived from each WZSP for 4 days, total RNA was extracted from the HEK293 cells and amplified *via* RT-PCR to detect the expression of PERV *gag*, *pol*, and *env* genes in the human cells. Overall, we screened 105 WZSPs, and 17 WZSPs tested negative in the assay.

Subsequently, the HEK293 cells cocultured with PBMCs derived from 8 out of 17 abovementioned WZSPs were maintained for a longer time, as were those derived from the positive control WZSP153 (a pig with transmitting PERV). During this period, genomic DNA as well as total RNA of the HEK293 cells were collected at each cell passage, followed by PCR and RT-PCR analysis for PERV detection. PERV infection of HEK293 cells was also determined by the measurement of reverse transcriptase (RT) activity in the HEK293 cell culture supernatant. Finally, 5 out of 8 WZSPs were confirmed to be negative for all these assays (**Figure 1** and **Table 1**). These 5 WZSPs could be considered to have non-transmitting PERV, that is, there was no *in vitro* transmission of PERV from pig to human cells.

Whole-Genome Resequencing of WZSPs With Non-Transmitting PERV

We conducted whole-genome resequencing on one of these non-transmitting pigs (i.e., WZSP452) to further investigate the sequence characteristics. We compared the quality control gene fragment with the reference genome (Wuzhishan Inbred Pig GenBank: AJKK01000000.1). The average depth was 13.55×, and these mapped reads covered 95.03% of the swine reference assembly. The genomes of WZSP452, Wuzhishan isolate L1-53 (GenBank No.: AJKK01000000.1) and Duroc isolate TJ Tabasco (GenBank No.: AEMK00000000.2) were searched for the *pol* gene of PERV-WZSP. In total, 95 *pol* gene fragments were detected in WZSP452, with 1 fragment longer than 3000 bp, 4 fragments between 1000 and 3000 bp, and the remaining fragments all shorter than 1000 bp. None of these *pol* gene fragments were intact. We also found a similar scenario in Wuzhishan isolate L1-53. In Duroc isolate TJ Tabasco, 49 *pol* gene fragments were detected, with 18 fragments longer than 3000 bp, 9 fragments

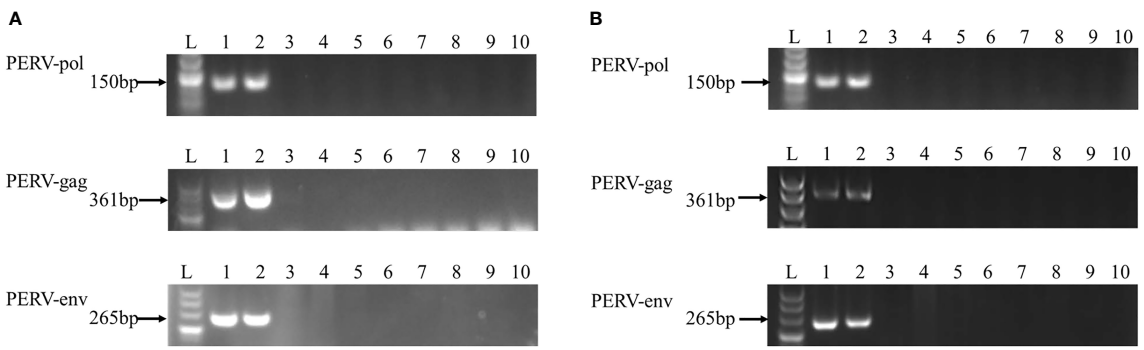

FIGURE 1 | PCR **(A)** and RT-PCR **(B)** analysis of PERV elements. Agarose gel electrophoresis was performed with PERV-pol, PERV-gag, and PERV-env PCR and RT-PCR products obtained from DNA **(A)** and RNA **(B)** prepared from HEK293 cells cocultured with the PBMC derived from the following WZSPs: lane 2, WZSP153 (a transmitting WZSP, as a positive control); lane 3-9, WZSP169, WZSP456, WZSP444, WZSP129, WZSP1213, WZSP505, WZSP452. Lane 1, positive control (PK15 cells); lane 10, no template control (NTC). The sizes of the products obtained are indicated to the left.

TABLE 1 | Reverse transcriptase (RT) activity in the supernatant of HEK293 cell cocultured with PBMC derived from WZSPs.

Pig No.	RT activity (μU/ml) in the supernatant of HEK293 cells on following days passaged																						
	Days post coculture with PBMC derived from WZSPs																						
	2	4	7	9	10	14	16	18	19	21	24	27	28	33	35	40	42	46	47	49	51	54	61
169	–		–			–				–			–		–		–			1450.74			
456				–					–			–		–		–			–			–	–
444				–					–			–		–		–			–			–	–
129	–		–					–		–			–		–		–				647.78		
1213				–					–			–		–		–			–			–	–
505	–		–			–				–			–		–		–	–					
452	–		–			–				–			–		–		–				–		
1209	–	–		–	–	–	–			421.18	445.81												
153				–					–			658.65		475.96									

Shown are data of RT activity in the supernatant of HEK293 cell cocultured with PBMC derived from WZSPs for approximately 24-61 days. Blank fields indicate that the RT activity was not tested on that day. "–" indicates that the RT activity was negative. 153 is a transmitting WZSP, which is used as the positive control.

between 1000 and 3000 bp and 22 fragments shorter than 1000 bp. Among them, 8 *pol* gene fragments were intact (**Table 2**).

We next searched the genome of WZSP452 for the nucleotide sequences of PERV-WZSP-encoding proteins (*gag*, *pol, env*) with the following parameters: e-value cutoff 1e-10, ≥80% sequence identity, and ≥80% length coverage. A total of 8 PERV-derived protein-coding genes were identified, including 1 *gag* gene, 2 *pol* genes and 5 *env* genes. Only 1 copy of the complete sequence of PERV, integrated into Scaffold5028, was detected in the genome of WZSP452 (**Table 3**), consistent with the results when screened with the complete genome of PERV reference sequence. The 2 assembly *pol* sequences were further validated by PCR and subsequent sequencing analysis. A sequence gap of 690 bp within the *pol* gene region of Scaffold640 was filled with Sanger sequence data generated with primers that flanked the gap. The complete *pol* gene of Scaffold640 was 3437 bp in length. Compared with the *pol* sequence of the PERV-WZSP genome, a 2-bp (CC) insertion after nt 3026 of Scaffold640-*pol* resulted in a frameshift mutation and premature termination at peptide position 1038. The *pol* gene of Scaffold5028 was 3360 bp in length and carried a 235A>T mutation, confirmed by next-generation sequencing, that led to a premature stop codon and resulted in a protein sequence truncated to 78 amino acids compared to the normal protein with 1144 amino acids (**Figure 2**).

These results indicated that all of the *pol* genes in the WZSP452 pig genome were defective. Furthermore, the *gag* and *env* genes of Scaffold5028 were defective as well, with lengths of 1574 and 1964 bp and premature termination at peptide positions 11 and 137, respectively.

Full-Length Transcriptome Analysis of WZSPs With Non-Transmitting PERV

WZSP452 pig transcriptomes were further sequenced using the PacBio platform to analyze the expression profile of PERV-*pol*. PacBio sequencing yielded a total of 29,8126 polished consensus reads. The mean length of all resulting transcripts was 2751 bp, ranging from 192 to 17,500 bp.

These transcripts were aligned to the genome of the PERV-WZSP isolate by BLAT search and screened against PERV

TABLE 2 | Copy number of PERV-*pol* gene in pig genomes.

Host	Copy number of PERV-*pol* gene fragments					
	Total copies	Fragments >3000 bp in length	Fragments between 1000 and 3000 bp in length	Fragments <1000 bp in length	Defective genes	Intact genes
Duroc isolate TJ Tabasco	49	18	9	22	41	8
Wuzhishan isolate L1-53	95	1	4	90	95	0
WZSP452	95	1	4	90	95	0

TABLE 3 | Location of PERV genome integrated into the WZSP452 genome.

WZSP452Scaffolds	Strand	Length	Start	End	Viral protein genes included
Scaffold12	–	2776	321642	318867	env
Scaffold42	–	2153	299876	297724	env
Scaffold226	+	3431	443655	447103	env
Scaffold640	+	6955	1381011	1387965	pol, env
Scaffold5028	+	8875	23882	32756	gag, pol, env

FIGURE 2 | Alignment of the deduced amino acid sequences of the PERV *pol* from Scaffold640 and Scaffold5028 with that of PERV-WZSP isolate (GenBank accession number: EF133960.1). The filled arrowhead indicates the position of a two base-pair (CC) addition in the sequence of Scaffold640, which results in a frameshift mutation and premature termination at peptide position 1038 (indicated by the filled triangle). The unfilled triangle indicates an A>T mutation in the sequence of Scaffold5028, which leads to a premature stop codon and resulted in a protein sequence truncated to 78 amino acids.

protein sequences by BLASTX with identity of ≥60% and length of ≥60 bp set as criteria. Then, the detected transcripts were aligned with the *pol* sequence of PERV-WZSP. Consequently, 23 transcripts that harbored the PERV-*pol* sequence were detected and named after their length (**Figure 3**). Of the 23 transcripts detected, 3 could be mapped to the full-length sequence of the *pol* gene. Sequence analysis further indicated that *pol*6825 revealed an A>G transversion at the first base of the start codon, leading to the loss of the start codon ATG (methionine) of the *pol* gene. In *pol*5723, we found a single base deletion at 209 nt causing a frameshift and a consequent premature stop codon at peptide position 70. In *pol*5141, the 23 nt deletion after the 1137 nt position led to an altered amino acid sequence and introduced a premature stop codon at peptide position 400.

The remaining 20 transcripts harbored incomplete *pol* gene sequences, and they were located as follows: two transcripts mapped from nts 60 to 3435 on the PERV-*pol* gene, and one of them had a deletion from nts 1802 to 3073; two transcripts mapped from nts 642 to 3434 and from nts 721 to 3435, respectively, and both of them had a deletion of nts 1802 to 3073; one transcript mapped from nts 1605 to 3435; one transcript mapped from nts 2336 to 3435; two transcripts mapped from nts 1 to 3208 with 2 deletions (nt 194-1414 and nt 1762-2561); eleven transcripts mapped from nts 3074 to 3435; and one transcript mapped from nts 3094 to 3435 with a deletion of nts 3156 to 3219.

These results indicated that all *pol* transcripts in the WZSP452 pig were unlikely to produce functional POL proteins.

DISCUSSION

To address the potential risk of PERV transmission in pig-to-human xenotransplantation, scientists have tried multiple approaches for years. During recent years, the successful generation of live piglets in which PERV genes were inactivated using CRISPR-Cas9 technology has attracted considerable attention. Undoubtedly, the generated PERV-inactivated pigs could be considered a remarkable breakthrough, as these pigs could serve as organ donors for future clinical xenotransplantation. This aside, using conventional animals (non-CRISPR/Cas9-treated animals) in well-controlled trials is believed to be feasible and has been performed in the past (10). Apart from pigs with the absence of PERV-C and low copy number and low expression of PERV-A and PERV-B, pigs that do not transmit human-tropic replication-competent PERVs (HTRC PERVs), namely, "non-transmitting pigs", could be an option.

In 2002, Oldmixon BA (33) investigated the PERV transmission characteristics of a unique inbred herd of miniature swine, with the result that inbred miniature swine that consistently do not transmit HTRC PERVs can be identified,

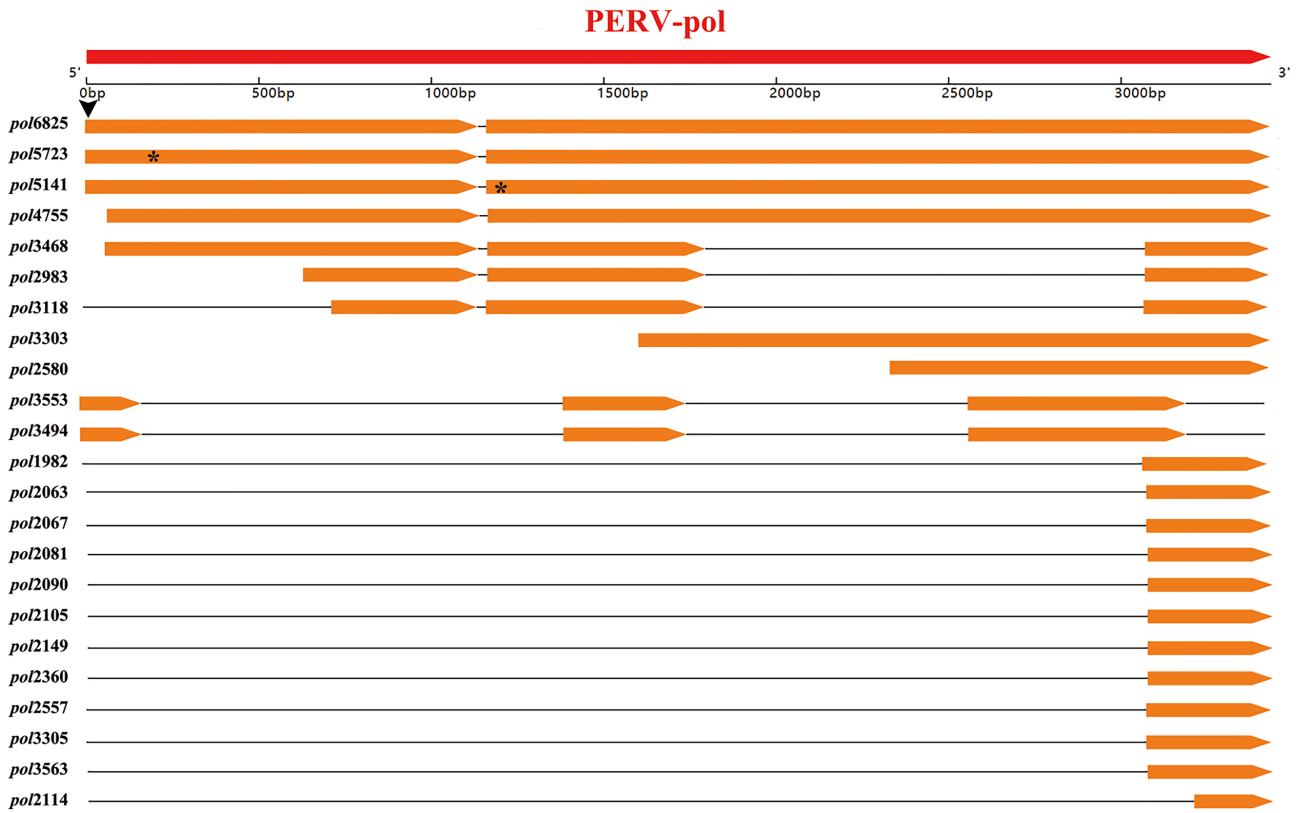

FIGURE 3 | Location of the detected 23 transcripts of WZSP425 on the PERV-WZSP *pol* gene. Arrow-rectangles in red indicated the ORF of PERV-WZSP *pol* gene, arrow-rectangles in orange indicated the detected 23 transcripts of WZSP452. Horizontal black lines represented deletions or introns of the gene. The black arrowhead indicated the position of a single base mutation (A>G), which results in the loss of start codon ATG of *pol*6825. The "*" symbol marked the positions of the premature stop codons in the *pol* gene from the *pol*5723 and *pol*5141.

and two of these lines of miniature swine (SLA d/d and SLA g/g) showed low incidences of transmission. In 2004, Wood JC (34) identified a group of miniature swine that do not carry PERV that infects either human or pig cells; these animals are referred to having a PERV-null transmission phenotype. Mainly using the coculture infectivity test with human HEK293 or porcine ST-IOWA target cell lines, Garkavenko O (35) established that Auckland Islands pigs have an extremely low risk of transmitting PERV infection and can therefore be qualified as "null" pigs. Islet cells from these well-characterized Auckland island pigs were used for two clinical trials that have been performed to treat diabetes in humans in New Zealand and Argentina (36, 37). In all cases, a positive medical effect was observed, and neither PERVs nor other porcine viruses under investigation were transmitted to the transplant recipients.

In the present study, we screened Chinese WZSP inbred pigs and found that 5 pigs consistently did not transmit HTRC PERVs in the *in vitro* coculture assays recommended by the U.S. Food and Drug Administration (FDA) (38). We further demonstrated that WZSP (WZSP452) was characterized by defective PERV *pol* genes at both the genomic DNA and transcriptome levels. To the best of our knowledge, this is the first report of a non-CRISPR/Cas9-treated Chinese miniature pig with defective porcine endogenous retrovirus *pol* genes. The WZSP inbred line in this study was developed based on the inbreeding of one male and one female WZSP by full-sib mating over more than 27 generations (39). These inbred WZSPs are characterized by a high level of homozygosity and genetic stability (40, 41). It has been demonstrated that genomic regions with extremely low rates of heterozygosis (<0.001%) account for 60% of the WZSP inbred line genome (40), providing a theoretical basis for massive gene loss during inbreeding. The defects in the PERV *pol* genes might be attributed to mutations, deletions, and insertions of proviral gene fragments accumulated during the long-term inbreeding process.

Further identification of more WZSPs with defective PERV *pol* genes and inbreeding might lead to the generation of a highly homozygous and genetically stable pig strain with defective PERV *pol* genes. This is a cost-effective strategy compared to gene editing combined with somatic cell nuclear transfer and, to some extent, would be safer. This strain could be a promising source of tissues and organs for xenotransplantation.

However, since the defective sites in different pigs could be different, we could not completely exclude the possibility that recombination and complementation of intact ORFs from two

defective proviral genomes could lead to infectious PERVs. Similarly, two defective RNAs can theoretically be packaged into a single viral particle and give rise to infectious virus by recombination and complementation, though the likelihood of such an event is considered to be low (42, 43). Therefore, further identification and monitoring of the PERV transmission characteristics of this pig strain are necessary.

In summary, the present study screened and identified the first non-CRISPR/Cas9-treated Chinese miniature pig with defective PERV pol genes. The development of a strain of highly homozygous and genetically stable pigs with defective PERV pol genes as source animal species is promising for xenotransplantation.

DATA AVAILABILITY STATEMENT

The whole genome sequence data and transcriptome data generated in this study have been deposited in NCBI Sequence Read Archive, under the accession number SRR6832872 and SRR10914223, respectively. The Sanger sequencing data of the pol sequence in Scaffold640 of WZSP452 have also been deposited at GenBank under the accession number MH198419.

ETHICS STATEMENT

The animal study was reviewed and approved by Beijing Experimental Animal Administration Committee.

AUTHOR CONTRIBUTIONS

YM, JJ, RF, and YL contributed equally. YM, JJ, RF, and YL performed most of the experiments and analyzed the data. YM and JJ wrote the paper. XZ, YZ, JY, and LMa collected samples and performed the experiments. YW, ML, and HY critically revised the manuscript. LMo and YD helped with result interpretation. SF and JZ conceived the project. All authors reviewed the draft before submission. All authors contributed to the article and approved the submitted version.

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Beneficial Impact of Interspecies Chimeric Renal Organoids Against a Xenogeneic Immune Response

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Background: Animal fetal kidneys have the potential to be used as scaffolds for organ regeneration. We generated interspecies chimeric renal organoids by adding heterologous rat renal progenitor cells to single cells from mouse fetal kidneys and applying the renal development mechanism of mouse fetuses to rat renal progenitor cells to examine whether rat renal progenitor cells can differentiate into renal tissues of the three progenitor cell lineages of kidneys between different species. Furthermore, we investigated whether chimeric renal organoids with an increased proportion of recipient cells reduce xenogeneic rejection.

Methods: C57BL/6JJmsSlc mice (B6 mice) and Sprague-Dawley-Tg (CAG-EGFP) rat (GFP rats) fetuses were used as donors, and mature male NOD/Shi-scid, IL-2RγKO Jic mice (NOG mice) and Sprague-Dawley rats (SD rats) were used as recipients. First, fetal kidneys were removed from E13.5 B6 mice or E15.5 GFP rats and enzymatically dissociated into single cells. These cells were then mixed in equal proportions to produce chimeric renal organoids *in vitro*. The chimeric organoids were transplanted under the renal capsule of NOG mice, and maturation of the renal tissues in the organoids was observed histologically. Furthermore, chimeric organoids were prepared by changing the ratio of cells derived from mouse and rat fetal kidneys and transplanted under the renal capsule of SD rats subjected to mild immunosuppression to pathologically analyze the strength of the xenogeneic immune response.

Results: The cap mesenchyme was reconstructed *in vitro*, and nephron progenitor cells and ureteric buds were mosaically comprised GFP-negative mouse and GFP-positive rat cells. In the *in vivo* environment of immunodeficient mice, chimeric renal organoids mosaically differentiated and matured into renal tissues of three lineages. Chimeric renal organoids with high rates of recipient rat cells showed milder rejection than complete xenograft organoids. The vessels of recipient rats entered from the periphery of the transplanted chimeric renal organoids, which might reduce their immunogenicity.

Conclusion: Interspecies chimeric renal organoids may differentiate into mature renal tissues of each renal progenitor cell lineage. Furthermore, they may reduce transplant rejection compared with xenograft organoids.

Keywords: organoid, chimera, regeneration, xenotransplantation, progenitors

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INTRODUCTION

In recent years, significant advancements have been made in human stem cell research and the generation of miniature organs such as brains, eye cups, and kidneys—known as "organoids,"—from human embryonic stem cells and induced pluripotent stem cells (iPSCs) *in vitro* has garnered much scientific attention (1). However, not all complex developmental mechanisms can be faithfully reproduced *in vitro*, and there remain some challenges that need to be overcome before the cells can mature into a tissue population known as an "organ." We aimed to generate a mature human kidney by injecting human renal progenitor cells into the nephrogenic zone (NZ) of a heterologous animal fetal kidney using the nephrogenic mechanism of the animal fetus (2–4). When animal fetal kidneys are dissociated into single cells and then reaggregated *in vitro* in a mouse model, renal organoids containing nephrons and collecting ducts are formed (5); this indicates that the cells comprising animal fetal kidneys already have the ability to form mature renal tissues. Furthermore, chimeric aggregates can be formed between different species by mixing chondrocytes from chicken and mouse fetal organs to generate organoids by reaggregating fetal-derived cells *in vitro* (6). Using this interspecies chimeric organoid phenomenon, we believe that it will soon be possible to generate kidneys with mature human renal tissue by aggregating organoids produced from fetal kidneys of different species such as pigs with renal progenitor cells derived from human iPSCs and applying the renal developmental signaling pathway of animal fetuses to human renal progenitor cells.

The kidney has three major progenitor cell types: nephron progenitor cells (NPCs), stromal progenitor cells (SPCs), and ureteric buds (UBs). In the NZ under the embryonic renal capsule, these three progenitor cells interact with one another to spontaneously differentiate into tissues of their own lineage (7). However, it is unclear whether the three progenitor cells of different species can actually form a developmental environment (niche) in the interspecies chimeric renal organoid and whether the three lineages—nephron, stroma, and collecting ducts—can differentiate and mature simultaneously. We previously reported the generation of chimeric kidneys with nephrons comprising 100% rat cells by transplanting heterologous rat kidney progenitor cells into the NZ of genetically engineered mice, in which six2-positive NPCs can be specifically removed by drugs, and replacing the mouse NPCs with rat NPCs using the mouse hind kidney as a scaffold (8). Furthermore, we reported that exogenous SPCs can differentiate into multiple types of renal stromal cells in the same species by transplanting mouse SPCs into the NZs of other mouse fetal kidneys (9). Therefore, in the future, it may be possible to generate kidneys with a higher chimerism rate that include the renal stroma in addition to the nephron. The immunogenicity of interspecies chimeric renal organoids with a high chimerism rate of human cells based on heterologous animal fetal kidneys compared with normal xenografts is of great importance for the clinical application of chimeric kidneys.

In this study, we first examined whether a heterologous chimeric nephrogenic niche can be generated *via* the *in vitro* association of single cells from mouse fetal kidneys and renal progenitor cells from rat fetal kidneys. Then, in immunocompromised mice, we assessed whether the heterologous nephrogenesis mechanism generates mature renal tissues of the abovementioned three lineages. Furthermore, we generated chimeric renal organoids with an increased proportion of recipient rat cells and investigated whether transplantation of the chimeric organoid into adult rats reduces xenogeneic immune rejection.

MATERIALS AND METHODS

Research Animals

The Institutional Animal Care and Use Committee and the Safety Committee for Genetic Recombination Experiments of Jikei University School of Medicine approved the protocols for animal experiments (permission numbers 2021-017, 2019-013, R1-68). The experiments were conducted in accordance with the Guidelines for the Appropriate Conduct of Animal Experiments (2006) of the Science Council of Japan. Every effort was made to minimize animal suffering. Pregnant female C57BL/6JJmsSlc mice (B6 mice), pregnant female Sprague-Dawley-Tg (CAG-EGFP) rats (GFP rats), and mature male Sprague-Dawley rats (SD rats) were purchased from SLC (Shizuoka, Japan). Mature male NOD/Shi-scid, IL-2RγKO Jic mice (NOG mice) were purchased from CLEA (Tokyo, Japan).

Single Cell Extraction From Fetal Kidneys

Single cells from mouse and rat fetal kidneys were obtained following a method (10) that was a partial modification of a method reported previously (11). In brief, pregnant E13.5 B6 mice or E15.5 GFP rats were anesthetized *via* the inhalation of isoflurane (2817774, Pfizer, New York, NY, USA). Fetuses were harvested and the maternal mouse or rat was immediately euthanized by injecting pentobarbital (120 mg/kg; Kyoritsu Pharma, Tokyo, Japan). Fetuses removed from the mother's womb were immediately decapitated and euthanized. Fetal kidneys were harvested from the decapitated fetuses under an operating microscope and collected in 1.5-ml tubes containing α minimum essential medium (MEMα; 12561-056, Thermo Fisher Scientific, Waltham, MA, USA). The tube was then centrifuged at 700 g for 3 min, the supernatant was removed, and 1 ml of accutase (AT104, Innovative Cell Technologies, San Diego, CA, USA) was dispensed. The sample was vortexed and incubated at 37°C for 5 min (repeated twice), and further manual pipetting and incubation was performed again for 5 min. The cell suspension was then centrifuged at 300 g for 5 min to remove the subsequent supernatant of accutase. The pellet was resuspended in a volume of 1 ml of MEMα with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 1% antibiotic-antimycotic (15240062, Thermo Fisher Scientific), and 10 μM Y2763 (257-00511, Wako, Osaka, Japan). In addition, the cells were passed through a 40 μm cell strainer (352340, Corning, NY, USA) to remove clumps of cells to obtain a single-cell suspension of mouse or rat cells. Cell counting of the single-cell suspensions was performed, and mouse and rat single-cell suspensions were placed on U-bottom 96-well low-cell-binding plates (174929, Thermo Fisher Scientific) in the cell ratios 6:0, 5:1, 3:3, 1:5, and 0:6 to a total of 2×10^5 cells/well. Finally, the plates were centrifuged at 1,000 rpm for four min and incubated at 37°C in an incubator.

In Vitro Culture of Renal Organoids

The single cells were confirmed to aggregate into spheroids by the next day (Day 1). After that, the medium containing Y27632 was removed, and the cells were cultured in medium containing MEMα, 10% FBS, and 1% antibiotic-antimycotic, with medium changes until Day 3. Spheroids were observed under an inverted microscope (Leica DMi1, Leica Microsystems, Wetzlar, Germany) or a fluorescence microscope (Olympus IX-71, Tokyo, Japan) and sampled on Day 4 or implanted under the renal capsule of NOG mice or SD rats. Specimens were whole-mount immunostained or frozen sectioned for fluorescent immunostaining or hematoxylin–eosin (HE) staining.

Transplantation of Renal Organoids Under the Renal Capsule

Renal organoids were transplanted under the renal capsule using a previously described method (10). The following is a brief description of the procedure. First, the recipient was anesthetized with isoflurane inhalation and a midline abdominal incision was made. The intestine was moved to the left or right side to expose the kidney, and the renal capsule in the lower part of the kidney was dissected at nearly 1 mm using a microshear. Then, the tip of the outer cylinder of the 22G surflo (SR-OT2225C, Terumo Corp, Tokyo, Japan) was cut at an angle. While the cut surface of the outer cylinder of the surflo was facing the renal parenchyma, it was inserted through the incision in the renal capsule to avoid damaging the renal parenchyma, and a small amount of saline water was placed under the renal capsule to detach a part of the renal capsule. After that, spheres in a 96-well plate were inhaled using the outer cylinder of the surflo with a syringe attached. The outer cylinder of the same surflo was inserted into the incision, and the renal organoids were transplanted under the renal capsule. The abdomen was then closed with a 5-0 thread. The recipients were mature male NOG mice or SD rats. Recipient SD rats received a small dose of an immunosuppressive drug (tacrolimus 0.3 mg/kg/day, Astellas Pharma, Tokyo, Japan), which was rejected when MNs of B6 mice were xenotransplanted into SD rats (12). At 14 days after transplantation, recipient NOG mice or SD rats were euthanized, and the transplanted renal organoids were subsequently collected and observed under a fluorescent stereomicroscope (Leica M205FA, Leica Microsystems, Wetzlar).

Whole-Mount Immunostaining, Immunostaining, and HE Staining of Frozen Sections

Renal organoid specimens for whole-mount immunostaining were fixed in 4% paraformaldehyde (163-20145, Wako) for 15 min at 4°C and then washed thrice with phosphate-buffered saline (PBS; 049-29793, Wako). Specimens were blocked for 1 hour at room temperature with 1% donkey serum (017-000-001, Jackson ImmunoResearch Laboratories, West Grove, PA, USA), 0.2% skim milk (190-12865, Wako), and 0.3% Triton X-100/PBS (25987-85, Nacalai Tesque, Kyoto, Japan) and were subsequently incubated with primary antibodies overnight at 4°C. After washing thrice with PBS, the samples were incubated with secondary antibodies conjugated with Alexa Fluor 488, 546, and 647 for 1 hour at room temperature. Specimens were mounted with SlowFadeTM Diamond Antifade Mountant with 4′,6-diamidino-2-phenylindole (DAPI; S36973, Invitrogen, Carlsbad, CA, USA). Specimens were observed under a fluorescence microscope (LSM880 confocal, Carl Zeiss). Specimens of renal organoids to be cryosectioned were fixed in 4% paraformaldehyde in PBS for 60 min and dehydrated in 20% sucrose in PBS. Specimens were embedded in OCT compound (4583, Sakura Finetek, Tokyo, Japan) and 8-μm thick frozen sections were prepared. HE staining was performed according to the standard procedures for histological analysis. Antigen activation for immunofluorescence staining was performed in a warm bath at 70°C for 20 min. After blocking for 1 hour at room temperature, the sections were washed thrice with PBS and then incubated with primary antibodies overnight at 4°C. The sections were then washed thrice with PBS and incubated with secondary antibodies conjugated with Alexa Fluor 488, 546, and 647 for 1 hour at room temperature. Afterward, the sections were then washed thrice with PBS and mounted using SlowFadeTM Diamond Antifade Mountant with DAPI and observed under an all-in-one fluorescence microscope (BZ-X800, Keyence, Osaka, Japan) or a fluorescence microscope (LSM880 confocal, Carl Zeiss). The primary antibodies used were as follows: chicken anti-GFP (ab13970, Abcam, Cambridge, MA, USA), rabbit anti-Six2 (11562-1-AP, ProteinTech, Rosemont, IL, USA), guinea pig anti-nephrin (GP-N2, Progen, Heidelberg, Germany), goat anti-megalin (sc-16478, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-E-cadherin (Ecad) (610181, BD, San Jose, CA, USA), rat anti-cytokeratin 8 (TROMA-I-C, DSHB, Iowa City, IA, USA), rabbit anti-platelet-derived growth factor receptor b (PDGFRb) (ab32570, Abcam), mouse anti-α SMA (A2547, Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-CD31 (ab28364, Abcam), rabbit anti-NKCC2 (SPC-401D, StressMarq Bioscience), mouse anti-aquaporin 2 (sc-515770, Santa Cruz Biotechnology), rabbit anti- V-ATPase B1/2 (sc-55544, Santa Cruz Biotechnology), goat anti-GATA3 (AF2605, R&D Systems), and goat anti-renin (AF4277, R&D Systems).

Measurement of the Chimeric Rate of the Constituent Cells of the Cap Mesenchyme

Immunostained sections of frozen organoids were photographed with a fluorescence microscope. The total number overall and the number of GFP-positive cells of Six2-positive NPCs and GATA3-positive UBs in each cap mesenchyme were counted under the same magnification, and the percentage of GFP-positive NPCs and GFP-positive UBs (%) was calculated by dividing the number of GFP-positive cells by the total number of cells. Two randomly selected sections from the renal organoid specimen were photographed at the same magnification, and a total of six images of each were made for analysis. This work was performed by two researchers.

Measurement of the Chimeric Rate of Each of the Three Renal Lineages in Chimeric Renal Organoids

Immunostained sections of frozen organoids were photographed with a fluorescence microscope. The total number overall and the number of GFP-positive cells of GATA3-positive cells as collecting ducts, Ecad-positive and GATA3-negative cells as nephrons, and PDGFRb-

positive cells as renal stroma were counted under the same magnification, and the percentage (%) of GFP-positive cells in each of the three lineages was calculated by dividing the number of GFP-positive cells by the total number of cells. Two randomly selected sections from the renal organoid specimen were photographed at the same magnification, and a total of six images of each were made for analysis. This work was performed by two researchers.

Measurement of the Number of CD3 Positive Cells Per Unit Area

Immunostained sections of each frozen organoid were photographed with a fluorescence microscope. The number of CD3-positive cells per unit area (pcs/mm^2) was calculated by counting the total number of CD3-positive T lymphocytes under the same magnification and dividing the total number of cells by the area. Two randomly selected sections from two specimens of renal organoids were photographed at the same magnification, making a total of six images each for analysis. This work was performed by two researchers.

Measurement of the Number of Nephrons Per Unit Area

HE-stained sections of organoid specimens of each cell ratio were photographed under the all-in-one fluorescence microscope to determine the number of glomeruli and area of organoids. The number of glomeruli per unit area (pcs/mm^2) was then calculated by dividing the number of glomeruli by the area of the organoid. Two specimens from each group of renal organoids of each cell ratio were taken, and three randomly selected sections were photographed at the same magnification for a total of six images in each group for subsequent analysis. This measurement process was performed by two researchers.

Statistical Analysis

Data were expressed as the mean ± standard error of the mean and analyzed using the Kruskal–Wallis test with a *post-hoc* test for comparison. All statistical analyses were performed using the Prism 8 software, and a p-value of <0.05 was considered significant.

RESULTS

In Vitro Reconstruction of the Nephrogenic Niche in Interspecies Chimeric Renal Organoids

Fetal kidneys of B6 mice and GFP rats were subjected to enzymatic treatment to obtain single cells. Single mouse cells were mixed with single rat cells in the same proportion to create chimeric cell aggregates *via* centrifugation on Day 0. On Day 1, chimeric spheroids of B6 mice and GFP rats were formed (**Figure 1A**). Next, we observed whether the nephrogenic niche of the interspecies chimeric renal organoids was reconstructed by whole-mount immunostaining on Day 3 (**Supplemental Figure 1**). In the renal organoids of mice only, a cap mesenchyme was reconstructed in which six2-positive and GFP-negative mouse NPCs aggregated around cytokeratin 8 (CK8)-positive and GFP-negative mouse UBs (**Figure 1B**, left). In GFP rat-only organoids, cap mesenchyme was reconstructed with six2-positive and GFP-positive rat NPCs aggregated around GATA3-positive and GFP-positive rat UBs (**Figure 1B**, right). In the mouse-rat chimeric renal organoids, the cap mesenchyme was also reconstructed, and NPCs and UBs were composed of GFP-negative mouse cells and GFP-positive rat cells in a mosaic pattern (**Figure 1B**, middle). In addition, immunostaining of frozen sections was used to verify the extent to which GFP-positive rat renal progenitor cells contributed to the cap mesenchyme. The total number of Six2-positive NPCs was 55.2 ± 12.1, the number of GFP-positive and Six2-positive NPCs was 18.0 ± 4.3, and the GFP-positive rate of NPCs was 34.5 ± 6.7%. The total number of GATA3-positive UB cells was 15.5 ± 1.7, the number of GFP-positive and GATA3-positive UB cells was 8.0 ± 2.8, and the GFP-positive rate of UB was 45.6 ± 14.6% (**Figure 1C**).

In Vivo Differentiation of Interspecies Chimeric Renal Organoids Into Three Renal Progenitor Cell Lineages

Chimeric renal organoids—a mixture of single cells from the fetal kidneys of B6 mice and GFP rats in the same proportion—were transplanted under the renal capsule of adult NOG mice—an *in vivo* environment—for further maturation (**Figure 2A** and **Supplemental Figure 2**). The specimens were collected on day 14 because the chimeric organoids gradually became disrupted by their own hydronephrosis after long-term transplantation beyond day 14. At the time of retrieval after 14 days, the chimeric renal organoids expressed GFP and were invaded by GFP-negative blood vessels derived from the recipient mice (**Figure 2B**, yellow arrowhead). Cryosections of the chimeric renal organoids showed extensive GFP expression, and HE staining revealed luminal structures (**Figure 2C**). Moreover, fluorescence immunostaining of the cryosections showed that the whole chimeric renal organoids comprised GFP-positive rat cells and GFP-negative mouse cells, with Ecad-positive tubules (**Figure 2D**), nephrin-positive glomeruli, and PDGFRb-positive renal stroma (**Figure 2E**). In GATA3-positive and Ecad-positive collecting ducts comprising GFP-positive rat cells and GFP-negative mouse cells were also observed (**Figure 2F**). Aquaporin 2-positive, GATA3-positive, GFP-positive rat principal cells, (**Figure 2G**) and V-ATPaseB1-positive, GATA3-positive, and GFP-positive rat intercalated cells (**Figure 2H**) were observed, forming luminal structures with GFP-negative mouse cells. Ecad-positive and GFP-positive rat distal tubules were connected to the GFP-negative and CK8-positive mouse collecting ducts (**Figure 2I**). An NKCC2-positive loop of Henle comprising GFP-positive rat cells and GFP-negative mouse cells was observed (**Figure 2J**). A megalin-positive and GFP-positive proximal tubule forming a lumen with GFP-negative mouse cells was also observed (**Figure 2K**). In the nephrin-positive glomeruli, podocytes comprised GFP-positive rat cells and GFP-negative mouse cells and PDGFRb-positive mesangial cells comprised GFP-positive rat cells and GFP-negative mouse cells (**Figure 2L**). PDGFRb-positive interstitial fibroblasts also comprised GFP-positive rat cells and GFP-negative mouse cells (**Figure 2M**). Furthermore, α SMA-positive vascular pericytes comprising GFP-positive rat cells were

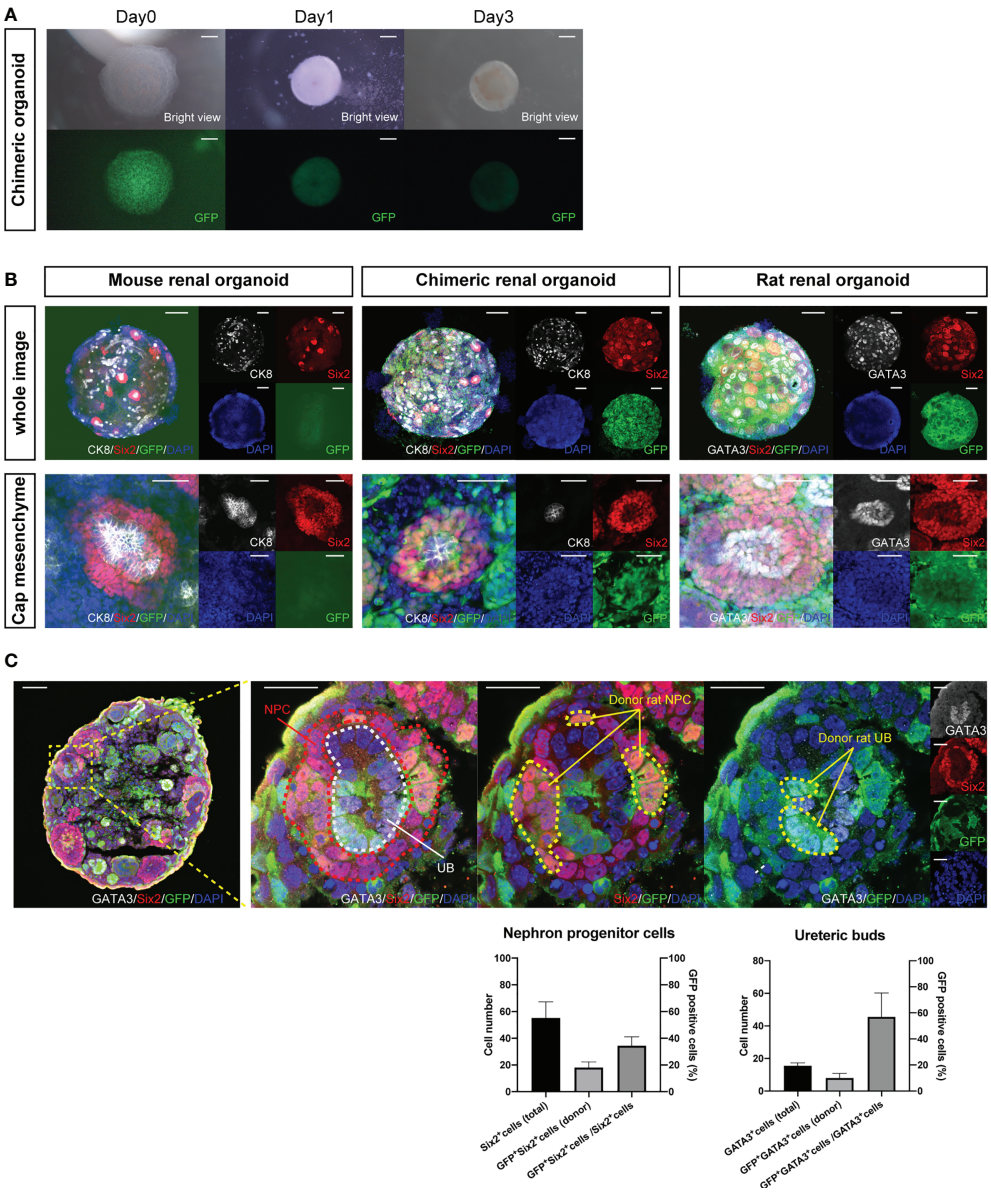

FIGURE 1 | Reconstructed nephrogenic niche in mouse-rat heterologous chimeras. **(A)** Images of changes over time in reaggregated spheroids of 1:1 mixtures of mouse and GFP rat fetal kidney cells, which aggregated into spheroids on Day 1. The cells aggregated into spheroids on Day 1. The expression of GFP in the spheroids was uniform and not polarized (scale bars 100 μm). **(B)** Whole-mount immunostaining of **(A)**. Multiple six2-positive cap mesenchymes were observed in mouse renal organoids, chimeric renal organoids, and rat renal organoids (scale bars in the upper column: 200 μm). In mouse renal organoids, six2-positive and GFP-negative NPCs reaggregated around CK8-positive and GFP-negative UBs. In rat renal organoids, six2-positive and GFP-positive NPCs aggregated around GATA3-positive and GFP-positive UBs. In chimeric renal organoids, both six2-positive NPCs and CK8-positive UBs were composed of GFP-positive rat cells and GFP-negative mouse cells (scale bars in the lower column: 50 μm). **(C)** Immunostaining of cryosections of chimeric renal organoids. Cap mesenchyme consisting of Six2-positive NPCs (red dotted line), GATA3-positive UBs (white dotted line) was reconstructed, and GFP-positive and Six2-positive donor rat NPCs, GFP-positive and GATA3-positive donor rat UBs were observed (yellow dotted line) (scale bar, left column: 50 μm, right column: 20 μm). The ratio of GFP-positive and Six2-positive cells to all Six2-positive cells and the ratio of GFP-positive and GATA3-positive cells to all GATA3-positive cells at the same magnification are shown as percentages. Error bars in the graphs represent the standard error of the mean (n=6).

observed (**Figure 2N**). In addition, as proof of organoid functionality, we observed GFP-positive renin-producing cells with endocrine functions (**Figure 2O**). We also examined the contribution of GFP-positive rat cells to each of the three lineages by counting the cells that were immunostained for representative cell markers. The percentage of GFP-positive cells in GATA3-positive collecting ducts was 66.1 ± 9.3%, that of GFP-positive cells in the tubules of Ecad-positive and GATA3-negative nephrons was 37.1 ± 9.4%, and that of GFP-positive cells in PDGFRb-positive stroma was 44.2 ± 6.7% (**Figure 2P**).

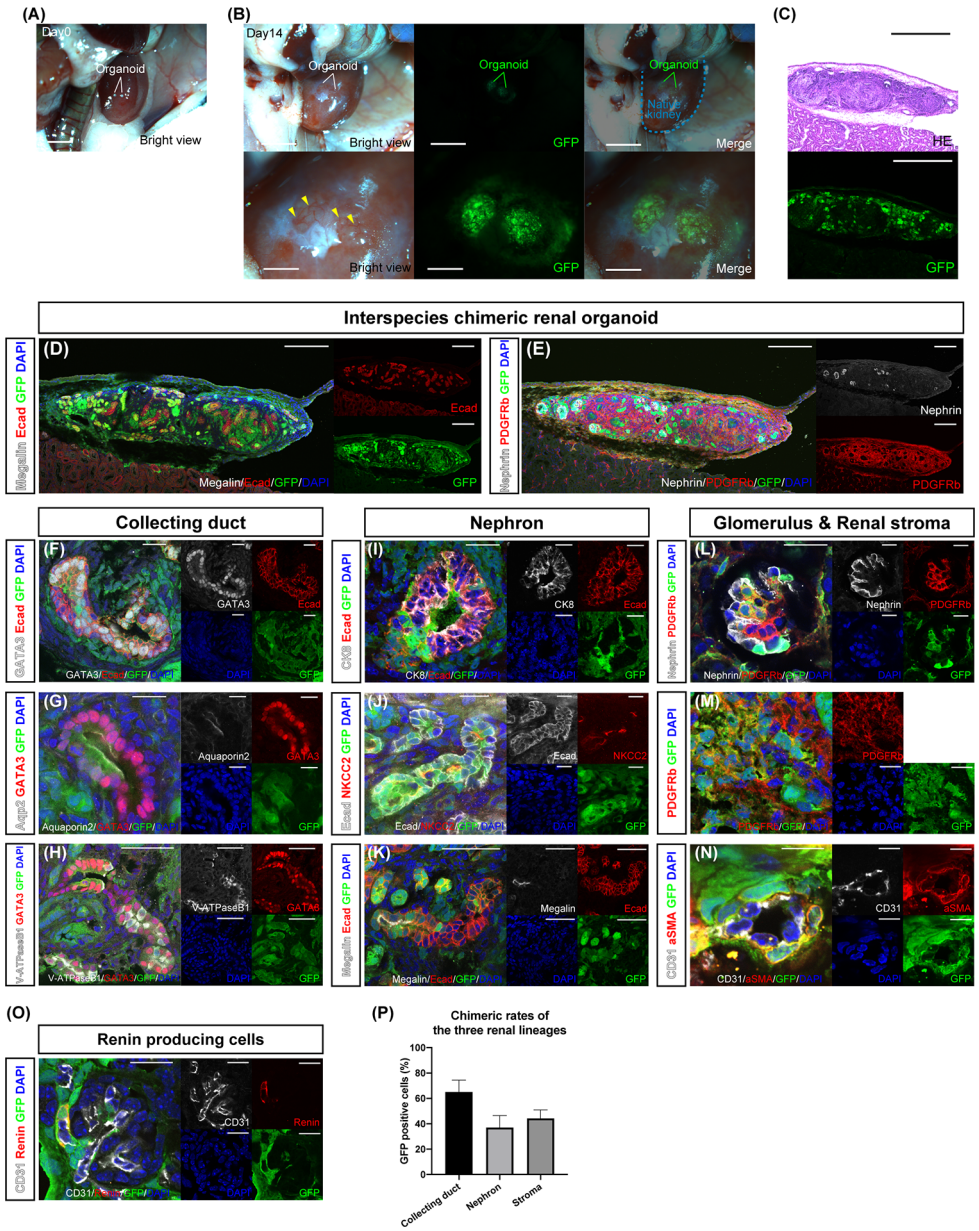

FIGURE 2 | *In vivo* differentiation of chimeric renal organoids into renal tissue. **(A)** Images of transplanted chimeric renal organoids made from mouse and rat fetal kidneys under the renal capsule of immunodeficient mice (scale bars 2 mm). **(B)** Brightfield and fluorescence images of chimeric renal organoids 14 days after transplantation (scale bars, upper column: 1 mm, lower column: 1 mm). Recipient-derived blood vessels invade the organoid (yellow arrowhead). **(C)** Fluorescence microscopy and HE staining images of frozen sections (scale bar 500 μm). **(D)** Fluorescence immunostaining image of a frozen section of the whole renal organoid (scale bar 200 μm). Multiple Ecad-positive tubular structures. **(E)** Nephrin-positive glomeruli and PDGFRb-positive renal stroma in the organoid (scale bar 200 μm). **(F)** GATA3-positive and Ecad-positive collecting ducts consisted of GFP-positive rat cells and GFP-negative mice cells (scale bar 20 μm). **(G)** Aquaporin 2-positive and GATA3-positive principal cells consisted of GFP-positive rat cells and GFP-negative mice cells (scale bar 20 μm). **(H)** V-ATPaseB1-positive and GATA3-positive intercalated cells consisted of GFP-positive rat cells and GFP-negative mice cells (scale bar 50 μm). **(I)** Connection of GFP-negative and CK8-positive mouse collecting ducts to GFP-positive and Ecad-positive rat distal tubules (scale bar 20 μm). **(J)** NKCC2-positive loop of Henle consisting of GFP-positive rat cells and GFP-negative mice cells (scale bar 20 μm). **(K)** GFP-positive and megalin-positive proximal tubules connected to the tubules of GFP-negative mice cells (scale bar 50 μm). **(L)** Nephrin-positive podocytes and PDGFRb-positive mesangial cells generated from GFP-positive rat cells and GFP-negative mice cells in the glomeruli (scale bar 20 μm). **(M)** PDGFRb-positive interstitial fibroblasts consisted of GFP-positive rat cells and GFP-negative mice cells (scale bar 20 μm). **(N)** α SMA-positive vascular pericytes from GFP-positive rat cells (scale bar 10 μm). **(O)** Renin-producing cells were found around the afferent arterioles of the glomeruli (scale bar 20 μm). **(P)** Contribution of GFP-positive rat cells to the three lineages of collecting ducts, nephrons, and stroma. The percentages of GFP-positive cells in GATA3-positive collecting ducts, Ecad-positive, and GATA3-negative nephron tubules, and PDGFRb-positive stroma are shown. Error bars in the graphs represent the standard error of the mean (each group, n = 6).

Reduction of Xenogeneic Rejection in Interspecies Chimeric Renal Organoid Transplantations

To investigate whether chimeric renal organoids reduce rejection compared with xenograft organoids, renal organoids were prepared *in vitro* by mixing cells from the fetal kidneys of B6 mice and SD rat-based GFP rats in various ratios (6:0, 5:1, 3:3, 1:5, and 0:6) and transplanted into the renal capsule of adult SD rats. The recipients were subjected to mild immunosuppression (tacrolimus 0.3 mg/kg/day) and organoids of the respective cell ratios were retrieved at 14 days after transplantation (**Supplemental Figure 3**). At the time of retrieval, 6:0 xenograft mouse renal organoids and 5:1 and 3:3 chimeric renal organoids were white and swollen, whereas 1:5 chimeric renal organoids and 0:6 allogeneic rat renal organoids were not swollen, showing vascular invasion from the recipient rat and strong GFP expression (**Figures 3A, B**). HE staining of the same tissue showed that the 6:0 xenograft mouse renal organoids and the 5:1 and 3:3 chimeric renal organoid tissues were thick and swollen with strong inflammatory cell infiltration and few glomeruli (**Figures 3C, D, F**). However, the 1:5 chimeric renal organoids, in which the cell ratio of the same rat as the recipient was increased, showed mild infiltration of inflammatory cells and mild abolition of glomeruli by inflammatory cells (**Figures 3C, D, F**). Furthermore, inflammatory infiltration was evaluated by immunostaining for CD3. Infiltration of CD3-positive cells was observed mainly in areas composed of xenogeneic, GFP-negative mouse cells, whereas areas generated from GFP-positive rat cells showed less infiltration of CD3-positive cells, and the remaining glomeruli were composed mainly of GFP-positive rat cells (**Figure 3E**). The number of CD3-positive cells per unit area was not significantly different in the 1:5 chimeric renal organoids compared to the control 0:6 allogeneic rat renal organoids, but the number of CD3-positive cells was significantly increased in the 6:0 xenograft renal organoids and the 5:1 and 3:3 chimeric renal organoids (**Figure 3G**).

DISCUSSION

In this study, we confirmed the chimeric establishment of rat renal progenitor cells within the mouse nephrogenic niche *in vitro* by mixing single cells of interspecies (mouse-rat) fetal kidneys. Furthermore, in the *in vivo* environment of immunodeficient mice, the mouse nephrogenesis mechanism could act on rat renal progenitor cells to generate chimeric renal organoids containing mature nephrons, collecting ducts, and stroma in a mosaic pattern comprising mouse and rat cells. We also demonstrated that increasing the ratio of recipient-derived renal cells in chimeric renal organoids attenuated rejection.

In vitro chimeric renal organoids showed that NPCs and UBs, as well as SPCs surrounding NPCs, comprised GFP-positive rat cells and GFP-negative mouse cells (**Figure 1B**, middle). Transplantation into the ipsilateral renal membrane of immunodeficient mice allowed the generation of chimeric renal tissue containing all three lineages (**Figures 2D–N**). This result was consistent with that of studies on cell lineage analysis showing that NPCs differentiate into podocytes, proximal tubules, and distal tubules (13) and SPCs differentiate into interstitial fibroblasts, mesangial cells, and vascular pericytes (14) as well as of a study showing that UBs differentiate into collecting ducts (15). Recently, the generation of human renal organoids containing nephrons, renal stroma, and collecting ducts from human iPSCs *in vitro* using a single protocol has been reported (16). However, faithful reproduction of the renal developmental mechanism and human kidney from human iPSCs remains challenging (17). In the future, a method should be developed to accurately induce individual differentiation into each progenitor cell such as NPC, UB, and SPC and to subsequently induce each lineage into renal tissue. NPCs and UBs differentiated from human mesenchymal stem cells or human iPSCs can reportedly be mixed with single cells from fetal mouse kidneys to facilitate the crossing of the heterogeneous barrier and differentiation into single lineage tissues such as nephrons and collecting ducts (18–20). Our results showed that not only differentiation into a single lineage but also the simultaneous interspecies generation of all three progenitor cell lineages of renal tissue, including the stroma, is possible. Because the gap in developmental signals is considered relatively small even between different species during organogenesis at the time of fetal kidney formation (21, 22), the present phenomenon indicates that signals related to the nephrogenesis of NPCs, UBs, and SPCs can be shared among different species. These results indicate that animal fetal kidneys have the ability to generate mature renal tissue between different species, which can be used for organ regeneration.

We previously verified suitable transplantation sites for the development of fetal and adult kidneys. For fetal kidney transplantation, we have shown that the periaortic region is superior for development in a small animal model of rats (23). In preclinical xenotransplantation of adult kidneys from pigs to monkeys, we have shown that orthotopic transplantation is superior (24). For the transplantation of renal organoids, we have previously developed a simple method of transplanting them under the renal membrane without damage (10). In the present study, we transplanted them under the renal capsule of the host and could generate mature renal tissues between different species.

Xenotransplantation has been garnering attention as a promising solution to the shortage of transplanted organs. It has already been demonstrated that long-term life support is possible in porcine-to-primate kidney transplantation (25). However, because strong immunosuppression is sometimes required to cause infection and death of the recipient, modification of the recipient by bone marrow chimeras (26) and attempts to reduce immunogenicity are required for clinical application in humans (27). The results of the present study show that chimeric renal organoids with a high engraftment cell fraction have immunological advantages compared with complete heterologous organoids (**Figures 3A–E**). Regarding this chimeric phenomenon and attenuation of immune responses, a previous study has described a mouse model that showed the possibility of dispersing the number of reactive T-cell precursors on the recipient side by transplanting islet cells

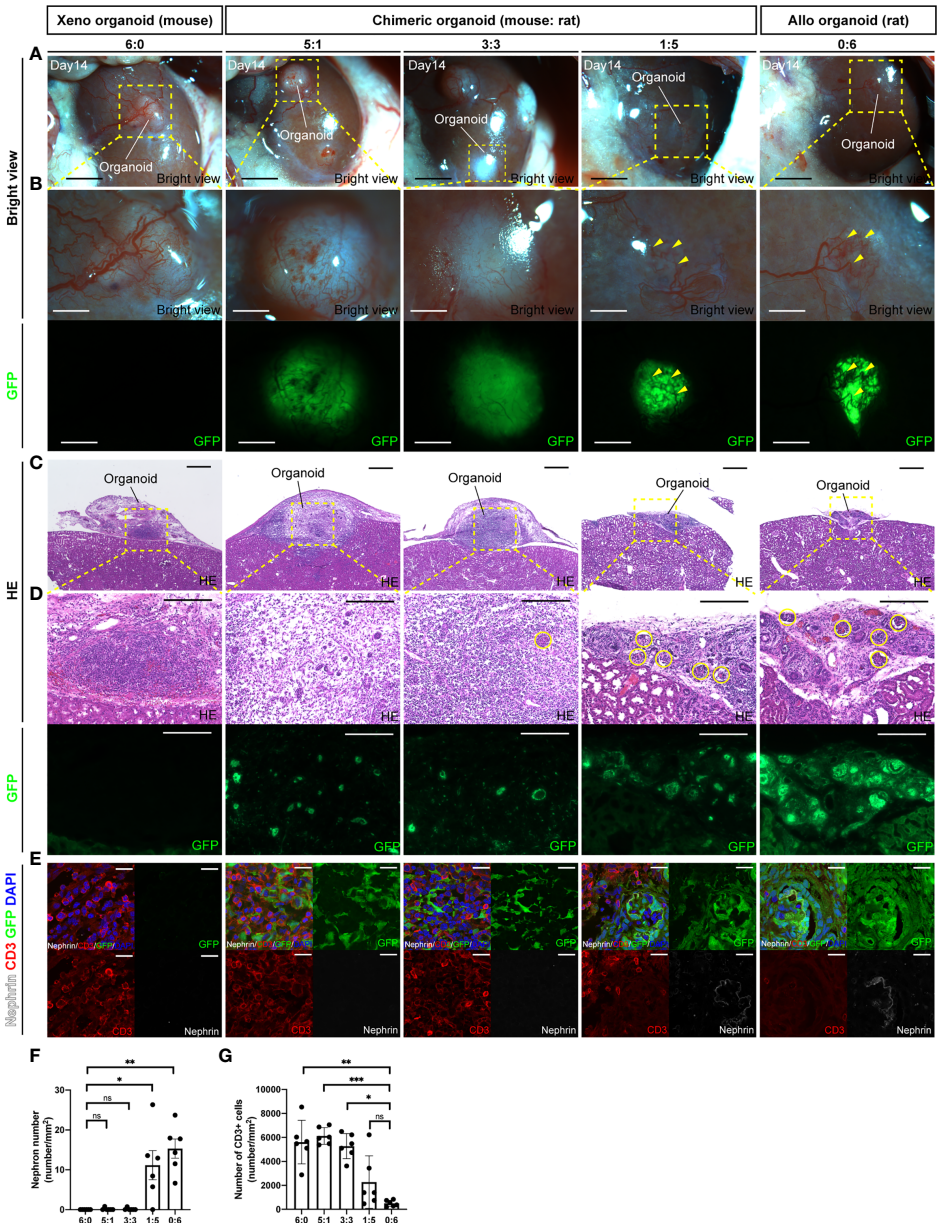

FIGURE 3 | Chimeric renal organoids reduce xenograft rejection. **(A)** Images of chimeric renal organoids generated from single cells of mouse and rat fetal kidneys at different cell ratios at 14 days after transplantation into the renal capsule of SD rats under mild immunosuppression protocol (scale bar 1 mm). **(B)** Magnified image of **(A)**. The 6:0 xenograft mouse renal organoids and the 5:1 and 3:3 chimeric renal organoids were white and enlarged, and the expression of GFP in the chimeric organoids was weak. The 1:5 chimeric kidney organoids showed the same vascular invasion of the recipient (yellow arrowhead) and strong GFP expression as the allogenic 0:6 rat renal organoids, the control (scale bars, upper column: 1 mm, lower column: 1 mm). **(C)** HE-stained images of each renal organoid. The 6:0 mouse renal organoids and 5:1 and 3:3 chimeric renal organoids were thickened and enlarged (scale bar 500 μm). **(D)** Magnified images of HE staining and GFP expression in each renal organoid. Both the xenogeneic mouse renal organoids and the chimeric renal organoids showed inflammatory cell infiltration and only a portion of the glomerulus remained (yellow circles). The 1:5 chimeric kidney organoids showed mild inflammatory cell infiltration and multiple glomeruli as in the control allogenic rat renal organoids (scale bars, upper column: 200 μm, lower column: 200 μm). **(E)** Images of CD3 immunostaining in each renal organoid. Xenograft 6:0 renal organoids, 3:3, and 5:1 chimeric renal organoids showed many CD3-positive cells and few glomerular structures. In 1:5 chimeric renal organoids, CD3-positive cells mainly infiltrated the region composed of GFP-negative heterologous mice, whereas renal tissue composed of GFP-positive rat cells showed less infiltration of CD3-positive cells (scale bar 20 μm). **(F)** The 1:5 chimeric renal organoids with a higher rat cell ratio had significantly higher glomerular counts compared to the 6:0 xenograft renal organoids (n = 6 in each group). Data are expressed as the mean ± standard error of the mean (Kruskal–Wallis test with *post-hoc* test). **(G)** The 1:5 chimeric renal organoids with a high rat cell ratio showed no significant difference in the number of CD3-positive cells compared to the 0:6 allogeneic renal organoids, but the 3:3, 5:1 chimeric renal organoids, and 6:0 xenograft renal organoids had significantly higher numbers of CD3-positive cells (n = 6 for each group). Data are expressed as the mean ± standard error of the mean (Kruskal–Wallis test with *post-hoc* test). *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.

derived from multiple donors (28) and a mouse model that leans the recipient's bone marrow cells toward the donor side by bone marrow transplantation (29). Furthermore, the phenomenon of reduced rejection while receiving immunosuppressive drugs has been clinically explained by the establishment of microchimerism (30). The fetal kidneys used as the cell source in this study have low expression of donor antigens that are subject to xenotransplant rejection (31). When used for transplantation, the vasculature is supplied by the recipient (32). In the present chimeric renal organoids, the vasculature also entered from the periphery (**Figures 2B**, **3A, B**), which may reduce their immunogenicity compared with that of normal adult kidney transplantation. From this immunological perspective, we are now focusing on the generation of chimeric kidneys with a higher chimerism rate derived from donor cells not only for the nephron, which can be regenerated by replacement of progenitor cells (8), but also for the renal stroma (9), collecting ducts, and ureter.

There are limitations to this study. First, this chimeric renal organoid is a closed system, and it will gradually be disintegrated by its own urine just like common renal organoids. Therefore, research on the connection to functional urinary tract components such as the ureter and bladder are necessary in the future. Next, in this study, mice and rats were relatively closely related animals for the chimera experiment. Pigs and humans, which we are considering in the future, are more distant from each other, and it may be difficult to create chimeras like this one. However, in addition to reports of the generation of interspecies chimeras by editing apoptotic genes into donor human cells (33), there are also reports of increased interspecies chimera rates by editing cell division and apoptosis-related genes into host cells (34), which may be applicable to our chimera technology.

Currently, we are investigating the generation of interspecies chimeric renal organoids using renal progenitor cells derived from human iPS cells and rodents that are more closely related species to each other than pigs. Once human-animal chimeric renal organoids are generated and mature human renal tissues can be generated from renal progenitor cells, they can be used not only for organ transplantation but also for various clinical applications such as drug sensitivity testing, disease models, and drug discovery using patient-derived or gene-edited iPS cells.

In conclusion, renal developmental niches were reconstructed between mouse and rat heterologous species, each capable of differentiating into renal tissue of three renal progenitor cell lineages. Furthermore, compared with xenotransplantation, interspecies chimeric renal organoids with a high chimerism rate of allogeneic cells could reduce xenogeneic rejection. These results will not only serve as a basis for future chimera technology to generate hybrid kidneys containing human renal tissue using animal fetal nephrogenesis but also help advance research on xenotransplantation and stem cells.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee and the Safety Committee for Genetic Recombination Experiments of The Jikei University School of Medicine.

AUTHOR CONTRIBUTIONS

YS, EK, SY, and TY designed the study. YS, with the help of NM, carried out the experiments. YS, with the help of NM, collected and analyzed the data. YS, with the help of EK and SY, wrote the manuscript. EK, SY and TY supervised the execution of the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.848433/full#supplementary-material

Supplementary Figure 1 | Schematic diagram of the experiments associated with **Figure 1**. *In vitro* generation of nephrogenic niches of interspecies chimeric renal organoids created by enzymatic treatment of fetal B6 mouse kidneys and fetal GFP rat kidneys into single cells, each mixed in equal proportions. RPC, renal progenitor cell.

Supplementary Figure 2 | Schematic diagram of the experiments associated with **Figure 2**. *In vivo* maturation of interspecies chimeric renal organoids under the renal capsule of immunocompromised mice, created by mixing fetal B6 mouse kidney and fetal GFP rat kidney into a single cell by enzymatic treatment, each in the same proportion.

Supplementary Figure 3 | Schematic diagram of the experiments associated with **Figure 3**. Renal organoids mixed with cells from the fetal kidneys of B6 mice and GFP rats in various ratios (6:0, 5:1, 3:3, 1:5, and 0:6) were prepared *in vitro*, transplanted into SD rats under mild immunosuppression, and the grafts were collected 14 days later.

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REVIEW
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Current Barriers to Clinical Liver Xenotransplantation

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Preclinical trials of pig-to-nonhuman primate liver xenotransplantation have recently achieved longer survival times. However, life-threatening thrombocytopenia and coagulation dysregulation continue to limit preclinical liver xenograft survival times to less than one month despite various genetic modifications in pigs and intensive pharmacological support. Transfusion of human coagulation factors and complex immunosuppressive regimens have resulted in substantial improvements in recipient survival. The fundamental biological mechanisms of thrombocytopenia and coagulation dysregulation remain incompletely understood. Current studies demonstrate that porcine von Willebrand Factor binds more tightly to human platelet GPIb receptors due to increased O-linked glycosylation, resulting in increased human platelet activation. Porcine liver sinusoidal endothelial cells and Kupffer cells phagocytose human platelets in an asialoglycoprotein receptor 1-dependent and CD40/CD154-dependent manner, respectively. Porcine Kupffer cells phagocytose human platelets *via* a species-incompatible SIRPα/CD47 axis. Key drivers of coagulation dysregulation include constitutive activation of the extrinsic clotting cascade due to failure of porcine tissue factor pathway inhibitor to repress recipient tissue factor. Additionally, porcine thrombomodulin fails to activate human protein C when bound by human thrombin, leading to a hypercoagulable state. Combined genetic modification of these key genes may mitigate liver xenotransplantation-induced thrombocytopenia and coagulation dysregulation, leading to greater recipient survival in pig-to-nonhuman primate liver xenotransplantation and, potentially, the first pig-to-human clinical trial.

Keywords: liver xenotransplantation, thrombocytopenia, xenograft, immune rejection, porcine

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INTRODUCTION

End-stage organ failure currently plagues over 106,000 people in the United States (1). The only definitive treatment for this devastating condition is organ transplantation. Unfortunately, the demand for organs outpaces the supply, a scenario which has created a severe organ shortage. The inevitable reality is that many on the transplant waiting list will die before ever receiving a life-saving organ. In order to address these issues, the practice of xenotransplantation, i.e., transplanting organs from one species to another, was explored. Over the past few decades, the pig was identified as the ideal organ donor for transplantation into humans based on its current use as a source of food, rapid

developmental timeline, relatively large litter size, roughly similar solid-organ size match, reasonable genetic similarity to humans, and its ability to be genetically-engineered (2). With the accessibility of genetically modified pigs, remarkable success has been achieved in pig-to-nonhuman primate (NHP) models. In a genetically modified pig-to-NHP model, life-supporting renal and heart xenografts have been reported to survive up to 435 days (3) and 195 days (4), respectively. A non-life-supporting pig-to-NHP heart xenograft survived for almost three years (945 days) (5).

Despite such promising advances in the field, the success of liver xenotransplantation has historically lagged behind its solid-organ counterparts. In contrast to the extended lifespan of heart and kidney xenografts, liver xenograft survival to date has been limited to less than one month (6, 7). Given the relatively shorter survival time of liver xenografts, the current potential clinical application of it is limited to a functional bridge to allotransplantation (8, 9). The primary barriers to successful liver xenotransplantation have consistently been the severe and rapid thrombocytopenia (10, 11) combined with uncontrollable coagulation dysregulation (11–13) that inevitably results in fatal hemorrhage of the recipient (9, 11, 13). The resolution of these major barriers may allow for the first pig-to-human liver xenotransplantation clinical trials as a bridge to allotransplantation, and potentially as definitive therapy for end-stage liver disease. We here provide a comprehensive review of the current status of pig-to-NHP liver xenotransplantation and the fundamental biological mechanisms of liver xenotransplantation-induced thrombocytopenia and coagulation dysregulation. Genetic modifications targeted at overcoming each of these obstacles will additionally be discussed.

CLINICAL AND PRECLINICAL EXPERIENCES IN LIVER XENOTRANSPLANTATION

Wild-Type (WT) Pigs

Prior to produce of genetically-engineered pigs, (WT) pig livers were used in pig-to-NHP liver xenotransplantation. Survival times were limited, and recipient demise as a result of sequelae from hyperacute rejection was inevitable. If the recipient managed to evade the lethality of hyperacute rejection, coagulopathy followed by uncontrollable hemorrhage rapidly ensued (14). The first and only attempt at pig-to-human clinical liver xenotransplantation was performed by Makowka et al. (15) in 1995. The case has been described in extensive detail elsewhere (13, 15); however, it is of historical significance that this first attempt was made with a liver from a WT pig (15).

Abbreviations: ASGR1, asialoglycoprotein receptor-1; ATG, anti-thymocyte globulin; GTKO, alpha galactosyltransferase gene knockout; hPCC, human prothrombin complex concentrate; HXR, hyperacute xenograft rejection; LSEC, liver sinusoidal endothelial cell; mAb, monoclonal antibody; NHP, nonhuman primates; KC, Kupffer cell; SIRPα, signal regulatory protein alpha; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TTC, Thrombin-thrombomodulin complex; vWF, von Willebrand Factor.

Genetically Modified Pigs

The advent and widespread use of gene-editing tools renewed interest in liver xenotransplantation. Initial genetically engineered pig-to-NHP models targeted the complement cascade, in particular the expression of humanized complement regulatory proteins such as CD55 (also known as decay-accelerating factor, or DAF) in pigs (16, 17). By modulating the complement cascade, hyperacute antibody-mediated rejection could theoretically be ameliorated. In combination with immunosuppressive therapy, two baboon recipients survived for more than four days, surpassing all previous attempts with WT pig livers (16). The ability to genetically optimize pig organs to improve recipient survival was no longer a dream: it was the future of liver xenotransplantation.

Since the initial successful liver xenotransplantation preclinical trials using genetically modified pigs, there has been an explosion in the number of genetically modified pigs available for use in xenotransplantation research (18). Genetic modifications targeted at xenoantigens, complement pathways, and components of the coagulation cascade have been performed in order to increase graft survival times and have been tested in pig-to-NHP models (19, 20) (**Table 1**). Prior to 2016, survival times in preclinical liver xenotransplantation were limited to just over two weeks (**Table 1**). In 2016, Shah et al. (6) broke through this plateau by performing an orthotopic pig-to-baboon liver xenotransplantation in which the recipient survived a total of 25 days (6, 22). Key differences from prior attempts included the continuous infusion of human prothrombin complex concentrate (hPCC) aimed at mitigating coagulopathy as well as the addition of belatacept, a monoclonal antibody (mAb) directed against CD80/86, a receptor involved in T-cell costimulation (6, 22). A year later, the same group replaced belatacept with an anti-CD40 mAb as the primary agent for costimulation blockade, with all other parameters held constant. The result was an astounding survival time of 29 days in one of the recipients (7). The livers in these trials were from α-galactosyltransferase gene-knockout (GTKO) pigs with no additional genetic modifications. Combining multiple genetic modifications with promising new pharmacological strategies could potentially pave the way to prolonged recipient survival.

In 2020, Dou et al. performed a 'heterotopic' pig-to-monkey liver xenotransplantation with splenectomy to achieve a 26-day of survive periods (personal communication by Dr. Kefeng Dou at the 2021 IXA meeting). The organ source pig was genetically modified to lack expression of key xenoantigens and to express complement regulatory proteins, coagulation cascade proteins, as well as several other genes (**Table 1**). In addition, certain modifications to the engraftment procedure were performed, including transection of the recipient inferior vena cava with subsequent end-to-end anastomoses from the donor portal vein to the distal inferior vena cava, donor hepatic vein to the proximal inferior vena cava, and donor hepatic artery to the recipient abdominal aorta. The donor biliary tract was anastomosed to the recipient jejunum. In this study, anti-thymocyte globulin (ATG) and anti-CD40mAb were used as immunosuppressive therapy.

TABLE 1 | Genetically-engineered pig-to-nonhuman primate liver xenotransplantation.

Year	Genetic Modifications	Recipient	Transplant	N	Pharmacologic Regimen	Survival	Reference
2000	hCD55	Baboon	Orthotopic	2	CyP CsA Cs	96, 192 h	(16)
2005	hCD55.CD50.HT	Baboon	Orthotopic	5	CyP CsA Cs Daclizumab Rituximab MMF	13, 18, 20, 21, 24 h	(17)
2010	GTKO	Baboon	Orthotopic	2	Cs MMF ATG Tacrolimus	3, 144 h	(10)
2010	GTKO.hCD46	Baboon	Orthotopic	5	Cs MMF ATG Tacrolimus	3, 20, 24, 96, 120 h	(10)
2010	GTKO.hCD46	Baboon	Orthotopic	3	CyP Cs MMF Tacrolimus	144, 144, 168 h	(10)
2012	GTKO	Baboon	Orthotopic	2	Cs ATG Tacrolimus CVF AZA anti-CD154 LoCD2b (one case)	144, 216 h	(19)
2012	MGH MS, GTKO	Baboon	Orthotopic	3	Cs ATG Tacrolimus CVF AZA anti-CD154 LoCD2b	6, 8, 9 days	(19)
2014	MGH MS, GTKO	Baboon	Heterotopic	3	Cs ATG Tacrolimus CVF	6, 9, 15 days	(20)
2015	WZ MS, GTKO	Tibetan monkey	Heterotopic	3	Cs MMF ATG Tacrolimus CVF anti-CD154 salviae miltiorrhizae	2, 5, 14 days	(21)
2016	MGH MS, GTKO	Baboon	Orthotopic	6	Cs ATG Tacrolimus CVF hPCC	1, 3, 4, 4, 6, 7 days	(22)
2016	MGH MS, GTKO	Baboon	Orthotopic	1	Cs ATG Tacrolimus CVF hPCC Belatacept	25 days	(6)
2017	MGH MS, GTKO	Baboon	Orthotopic	4	Cs ATG Tacrolimus CVF hPCC anti-CD40mAb	25, 5, 8, 29 days	(7)
2020	GTKO, CMAH-KO, B4GALNT2-KO, PERV-KO, hCD46, hCD55, hCD59, hTHBD, hTFPI, hCD39, hB2M, HLA-E, hCD47-TG	Rhesus monkey	Heterotopic	1	ATG anti-CD40mAb	26 days	Dou, personal communication

ATG, anti-thymocyte globulin; AZA, azathioprine; B4GALNT2, Beta-1,4-N-Acetyl Galactosaminyltransferase 2; CMAH, Cytidine monophospho-N-acetylneuraminic acid hydroxylase; Cs, corticosteroids; CsA, cyclosporine; Cyp, cyclophosphamide; GTKO, galactosyltransferase knockout; h, humanized; hPCC, human prothrombin complex concentrate; LoCD2b, rat anti-primate CD2 IgG2b; MGH MS, Massachusetts General Hospital miniature swine; MMF, mycophenolate mofetil; PERV, porcine endogenous retrovirus; WZ MS, Wu Zhanshen miniature swine.

The initial postoperative period was characterized by an initial depletion of recipient platelets followed by slight recovery, a stable hematocrit, and persistently low leukocyte levels due to ATG and anti-CD40mAb immunosuppressive therapy. At postoperative day (POD) 15, recipient liver enzymes began to climb, hinting at the first signs of possible graft rejection. In addition, total bilirubin continued to climb throughout the postoperative period, further indicating declining graft function. Graft synthetic function as measured by porcine albumin levels peaked at POD 4 and progressively declined thereafter, consistent with overall worsening graft function. Coagulation parameters, such as PT and aPTT, remained stable throughout the experiment. Despite encouraging laboratory findings, specimens taken at necropsy demonstrated focal hemorrhagic necrosis and thrombotic microangiopathy, consistent with ongoing coagulation dysregulation. Histopathologic specimens taken throughout the postoperative period demonstrated increasing IgG and IgM levels within the graft, consistent with an uncontrolled humoral xenogeneic response. Despite low total lymphocyte levels, analysis of lymphocyte subsets showed a significant increase in the number of $CD8^+$ T cells and $CD159a^+$ NK cells, consistent with ongoing and/or worsening cell-mediated rejection. In addition, substantial infiltration by recipient macrophages and neutrophils was noted within the graft. Taken together, these findings suggest that an integrated immune response involving both the innate and adaptive immune systems likely continues to exacerbate the consumptive coagulopathy and thrombocytopenia characteristic of species-discordant liver xenotransplantation.

The extensive preclinical experience in pig-to-NHP liver xenotransplantation to date has demonstrated the life-saving potential of liver xenotransplantation. With survival times approaching a month, clinical trials of liver xenotransplantation as a 'bridge' to allotransplantation are within reach (23). However, despite decades of work, the definitive combination of genetic modifications and pharmaceutical support required to bring liver xenotransplantation to the clinic continues to elude us due to the two persistent lethal barriers to liver xenotransplantation: 1) rapid, life-threatening thrombocytopenia and 2) uncontrolled consumptive coagulopathy, culminating in lethal hemorrhage.

THROMBOCYTOPENIA AND COAGULOPATHY AS MAJOR BARRIERS TO LIVER XENOTRANSPLANTATION

Thrombocytopenia

In early WT pig-to-NHP models, liver xenografts underwent rapid antibody-mediated rejection, leading to graft destruction and recipient demise within hours (9, 16). The consequences of thrombocytopenia thus took a back seat to the task at hand: overcoming hyperacute rejection. The advent of genetic engineering tools allowed for the establishment of GTKO pigs (24), the organs from which demonstrated drastically reduced hyperacute rejection in pig-to-NHP models (10, 25, 26). In 2010,

Ekser et al. (10) demonstrated that severe thrombocytopenia develops within 1 hour of pig-to-NHP orthotopic liver xenotransplantation from GTKO pigs transgenic for a human complement regulatory protein, CD46. The result was spontaneous hemorrhage in multiple organs in the absence of signs of humoral or cellular rejection, ultimately limiting survival times to a maximum of 7 days (10, 27). These observations demonstrated that severe, rapid thrombocytopenia occurred independently of organ rejection, a phenomenon that is not typical for heart or kidney xenotransplantation (13, 18). The conundrum prompted further research into the physiologic and immunologic mechanisms of liver xenotransplantation-induced thrombocytopenia. The current literature suggests severe thrombocytopenia following liver xenotransplantation results from 1) excessive platelet activation/aggregation and 2) aberrant platelet sequestration/phagocytosis.

Platelet activation classically occurs when platelet GpIb receptors bind von Willebrand Factor (vWF) on the exposed negatively charged surfaces of damaged vascular endothelium (**Figure 1**). The GpIb/vWF interaction induces platelet degranulation, releasing fibrinogen, vWF, serotonin, ADP, and Ca^{2+} into the bloodstream. Thromboxane A2 (TXA2) is released by damaged endothelial cells and promotes further platelet degranulation. ADP activates platelets, which causes a structural change that exposes GpIIb/IIIa receptors which then bind fibrinogen, resulting in the formation of a platelet plug. A finite number of platelets are consumed when this process occurs naturally.

In the setting of liver xenotransplantation, there are several points along this pathway that are altered as a result of donor-recipient interspecies incompatibilities. Schulte et al. (28) demonstrated that porcine vWF binds more tightly to human

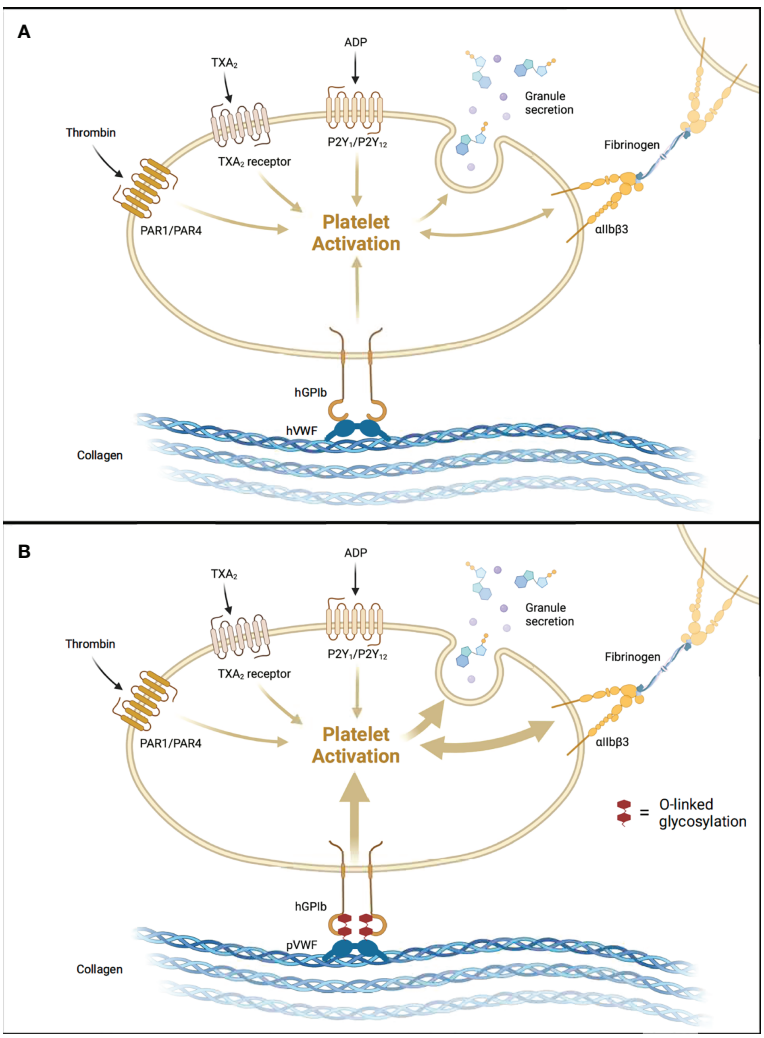

FIGURE 1 | Aberrant Activation of Human Platelets by Porcine vWF. **(A)** Human-to-human allogeneic platelet activation. **(B)** Pig-to-human xenogeneic platelet activation augmented by tighter pvWF-hGpIb binding. hGPIb, human glycoprotein Ib; hVWF, human von Willebrand Factor; pVWF, porcine von Willebrand Factor. Created with BioRender.com.

GpIb, resulting in significantly increased platelet activation *in vitro*. This tighter binding was shown to result from increased O-linked glycosylation in porcine vWF compared to human vWF (**Figure 1**) (28). In a subsequent *ex vivo* pig-to-NHP liver xenoperfusion model, human platelet activation, as measured by beta-thromboglobulin levels, was significantly higher in the xenoperfusion groups compared to alloperfusion controls, and the combined addition of anti-GpIb antibodies with DDAVP (desmopressin) slightly reduced platelet activation (not statistically significant) (29). Indeed, all xenoperfusion groups demonstrated significantly lower platelet counts when compared to alloperfusion controls. These findings suggest that porcine vWF/primate GpIb interspecies incompatibilities, specifically increased O-linked glycosylation, increase platelet activation and consumption, and ultimately contribute to liver xenotransplantation-induced thrombocytopenia. Recently, Connolly et al. (30) demonstrated that expression of human vWF in porcine livers significantly reduced platelet consumption in an *ex vivo* liver perfusion model, further supporting this notion.

The increased platelet activation in liver xenotransplantation additionally results in downstream amplification of platelet aggregation. As platelets degranulate, increased vWF and fibrinogen are released and subsequently bound by nearby activated platelets through GpIIb/IIIa receptors. The result is extensive platelet aggregation. This notion is supported by *in vitro* studies demonstrating that baboon platelet aggregation is induced by direct contact with porcine aortic endothelial cells, liver sinusoidal endothelial cells (LSECs), and hepatocytes (31). Baboon platelet aggregation was ameliorated when these porcine cell lines were pre-treated with anti-GpIb and anti-GpIIb/IIIa antibodies (31). Xenoperfusion studies conducted by Burdorf et al. (32) demonstrated anti-GpIb antibodies delayed the development of thrombocytopenia by 2 hours, supporting the notion that dysregulated platelet activation contributes to liver xenotransplantation-induced thrombocytopenia. These findings provide substantial evidence that disrupting components of platelet activation and aggregation reduces platelet consumption which subsequently mitigates the severe thrombocytopenia seen in liver xenotransplantation.

Although there is substantial evidence for increased platelet activation/aggregation in pig-to-NHP liver xenotransplantation models, other studies have also shown that platelet *sequestration* within the xenograft may play a critical role (8, 33). In an *ex vivo* perfusion model of human blood through a wild-type pig liver, 93% of platelets were removed within 15 minutes despite no evidence of endothelial cell or platelet activation (8, 33). Tissue biopsies of the liver xenograft showed extensive platelet phagocytosis by porcine Kupffer cells (KC), and *in vitro* co-culture assays demonstrated human platelet sequestration by porcine LSECs (33). Degraded human platelets were additionally observed inside porcine hepatocytes. These findings provide strong evidence for liver sequestration of recipient platelets by porcine LSECs, Kupffer cells, and hepatocytes as a key mechanism of liver xenotransplantation-induced thrombocytopenia.

Liver Sinusoidal Endothelial Cells

LSECs play a major role in scavenging bloodborne waste through an intrinsically elevated endocytic capacity and are therefore of particular interest in the setting of species-discordant liver xenotransplantation. Paris et al. (34) demonstrated that porcine LSECs recognize and bind the galactose β1-4 N-acetyl glucosamine (Galb1,4-NacGlc) glycoprotein on human platelets *via* asialoglycoprotein receptor-1 (ASGR1) (**Figure 2**). Elimination of this glycoprotein by treatment with asialofetuin proportionally decreased platelet phagocytosis by LSECs *in vitro* (34). Reduced human platelet phagocytosis in *ex vivo* perfusion through ASGR1-deficient pig livers has also been reported (33). ASGR1-mediated platelet phagocytosis is not specific to porcine LSECs, as it was shown to also occur in porcine aortic and femoral arterial vascular endothelium (11, 35), suggesting ASGR1-mediated platelet phagocytosis may be a generalized mechanism of species-discordant platelet consumption.

Porcine Kupffer Cells (pKC)

Porcine Kupffer cells (KC) also play a major role in liver xenotransplantation-induced platelet sequestration (9). Chihara et al. (36) demonstrated that CD18 is central to porcine KC recognition of human platelets *in vitro*. Anti-CD18 antibodies and siRNA knockdown of CD18 in pig cells resulted in significantly reduced human platelet binding and phagocytosis (36). The mechanism by which porcine KC CD18 recognizes and phagocytoses human platelets is thought to occur through the recognition of CD40 ligand (CD40L, aka CD154) on activated platelets (37) and β-N-acetyl d glucosamine (β-GlcNac) on cold-activated platelets (13, 36). Porcine KC additionally express CD40, which further augments recognition and phagocytosis of activated human platelets through CD40L. Indeed, monoclonal antibodies directed against the CD40/CD40L complex have demonstrated prolonged liver xenograft survival in pig-to-NHP models when used as part of a costimulation blockade regimen (7, 38). To date, genetic modifications to this axis have not been attempted, likely due to pig viability concerns. Taken together, these findings suggest CD18 and CD40 receptors on porcine KCs are crucial to human platelet recognition and phagocytosis and serve as a primary target for further research to prevent liver xenotransplantation-induced thrombocytopenia.

Macrophages, including porcine KC, predominantly mediate phagocytosis through the interaction of signal regulatory protein α (SIRPα) on the macrophage with the ubiquitously expressed CD47 on "self" cells, including platelets, red blood cells, and leukocytes. Appropriate species-specific binding of CD47 to SIRPα results in dominant inhibitory signaling through immunoreceptor tyrosine inhibitory motifs (ITIMs), leading to the prevention of phagocytosis by the macrophage (39). In the setting of pig-to-human or pig-to-NHP liver xenotransplantation, porcine SIRPα (pSIRPα) exhibits suboptimal binding to human CD47 (hCD47). Porcine KC were shown to phagocytose human platelets in a porcine SIRPα/human CD47-dependent manner *in vitro*, and transgenic expression of human SIRPα on porcine KC significantly reduced human platelet phagocytosis (13). These

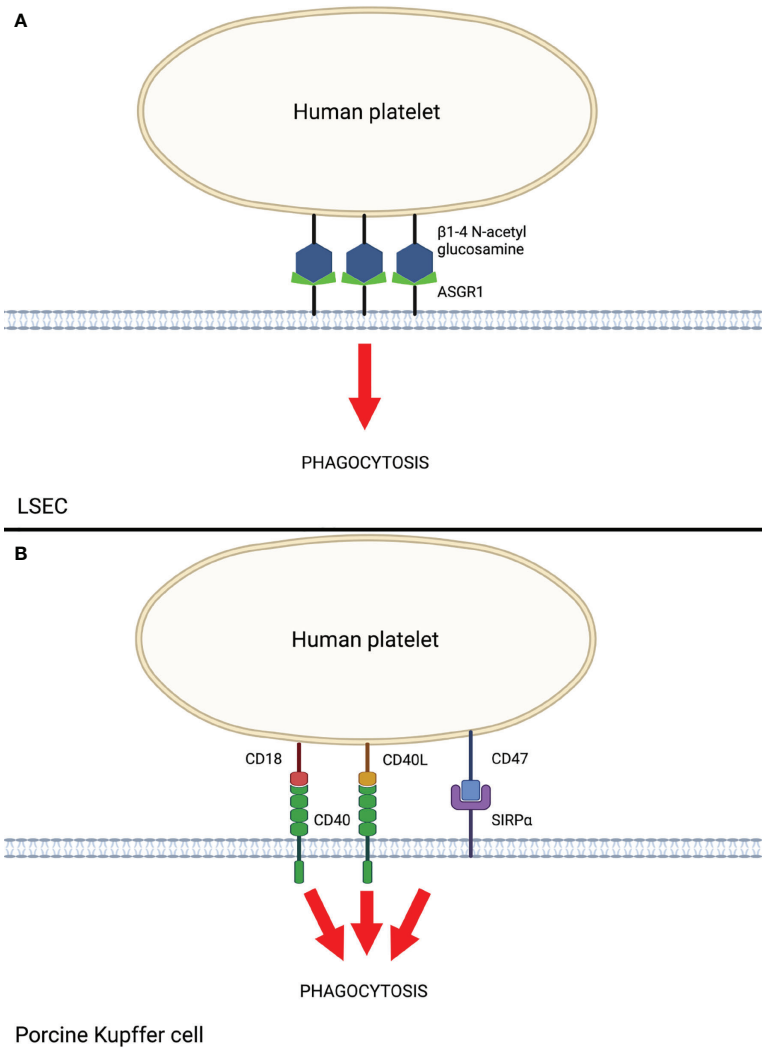

FIGURE 2 | Human Platelet Sequestration by Porcine LSEC and Kupffer Cells. **(A)** Liver sinusoidal endothelial cell (LSEC)-mediated phagocytosis of human platelets *via* ASGR1. **(B)** Porcine Kupffer cell-mediated phagocytosis of human platelets *via* interactions between CD40 and SIRPα with respective ligands on human platelets. Created with BioRender.com.

results provide evidence that genetic modification of organ-source pigs to express human SIRPα may reduce porcine KC-mediated platelet phagocytosis and subsequently mitigate liver xenotransplantation-induced thrombocytopenia.

Coagulation Dysregulation

Aberrant activation of the coagulation cascade is a hallmark feature of hyperacute rejection. In pig-to-NHP liver xenotransplantation, however, dysregulated coagulation occurs even in the absence of immunologic evidence of hyperacute rejection (13). Interestingly, coagulation dysregulation is a substantially greater barrier to liver xenotransplantation than heart or kidney xenotransplantation (11, 40).

Genetic discordance in tissue factor (TF) and subsequent constitutive activation of the extrinsic pathway is well-supported in the literature as a primary driver for liver xenotransplantation-induced consumptive coagulopathy (41, 42). TF is constitutively expressed in subendothelial fibroblasts and muscle cells and is inducibly expressed in endothelial cells in the setting of systemic inflammation (43). Upon exposure of TF to the bloodstream, it binds and subsequently activates Factor VII, initiating the extrinsic pathway of the coagulation cascade (11, 44). TF activity is regulated by tissue factor pathway inhibitor (TFPI), a polypeptide that binds the TF/VIIa complex and inhibits its activity. Molecular incompatibilities between donor TFPI and recipient TF have been a subject of intensive investigation. Lee et al. (45) initially demonstrated *in vitro* that both recombinant pig TFPI and human TFPI efficiently inhibited the activation of human Factor Xa by human TF/VIIa. More recently, Ji et al. (21) provided *in vitro* evidence that pig TFPI does *not* inhibit human TF as efficiently as

human TFPI, suggesting molecular incompatibilities between pig and human may, in fact, contribute to coagulation dysregulation in liver xenotransplantation. Additionally, this group demonstrated that recipient (baboon) TF is activated in pig-to-NHP heterotopic liver xenotransplantation, but donor (pig) TF remains inactivated (21), suggesting the aberrant upregulation of recipient TF combined with genetically incompatible donor TFPI is the key issue. Interestingly, Ahrens et al. (43) recently demonstrated that siRNA knockdown of porcine TF significantly increased clotting time and decreased thrombus formation when compared with WT pigs, suggesting porcine TF contributes to aberrant liver xenotransplantation-induced coagulation dysregulation despite initial evidence to the contrary (21). Preclinical trials involving human TFPI expressing pigs have shown promising results (Kefeng Dou, personal communication); however, the specific contribution of human TFPI as compared to the additional interventions in the trials remains to be clarified. Moreover, it is reported that overexpression of human TFPI on pig cells were compatible with spontaneous bleedings in pigs and eventual death (David Ayares, personal communication).

Species incompatibilities in the thrombin-thrombomodulin complex (TTC) have been proposed as an additional contributing factor to coagulation dysregulation in liver xenotransplantation. Thrombomodulin is an integral membrane protein expressed on the surface of endothelial cells and is a cofactor for thrombin. The interaction of thrombin with thrombomodulin results in the formation of the TTC, which through the activation of protein C inhibits factors Va and VIIIa, resulting in dampening of the coagulation cascade. At the same time, the removal of thrombin inherent to the formation of the TTC further reduces the number of procoagulant factors. As such, appropriate binding of thrombomodulin to thrombin is essential for regulation of the coagulation cascade. *In vitro* studies demonstrate that porcine thrombomodulin binds to human thrombin, but the resulting TTC fails to effectively activate human protein C (46). Transgenic expression of human thrombomodulin (hTM) in porcine aortic endothelial cells resulted in substantially greater activation of human activated protein C *in vitro* (47), providing promising evidence for the utility of hTM transgenic pigs. Organs from hTM transgenic pigs were shown to activate protein C at a substantially higher rate than wild-type pigs; however, this expression was the lowest in the liver (48). Further optimizing hTM expression in porcine livers is necessary to evaluate the true benefit of this genetic modification with respect to liver xenotransplantation, as shown in prolonged survival in preclinical hTM pig-to-NHP kidney (49) and heart (4, 50) xenotransplantation.

ADDITIONAL CONSIDERATIONS

Liver Xenograft Function

Overcoming the current immunologic and hematologic barriers to liver xenotransplantation is crucial, however evaluating whether the transplanted organ will function at a sufficient level to sustain human life is of equal importance. In 2010 the Pittsburgh Group evaluated a comprehensive set of liver xenograft functional parameters in a series of pig-to-baboon orthotopic liver xenotransplants, including porcine albumin, fibrinogen, haptoglobin, plasminogen, almost all coagulation factors, bilirubin, and all liver enzymes (51). Overall hepatic synthetic function was maintained throughout the post-operative period which was documented by the measurement of liver enzymes as well as INR. Although INR levels were acceptable following pig liver xenotransplantation in baboons, many coagulation factors in baboons remained significantly lower than pre-transplant indicating, in most cases, that they were adjusted with the level of porcine coagulation factors (51). Consistent findings were reported by Kim et al. in 2012, most notably the requirement for continuous IV infusion of albumin and the presence of significantly lower porcine coagulation factor levels (19). Taken together, these findings suggest porcine hepatic function was stable after xenotransplantation and closely approximates NHP hepatic function. Although one can argue about the relatively short survival of the recipient baboons in both studies (3 hours to 9 days), a recent study by Shah et al. (7) showed that two baboons survived almost one month with normal liver function following pig liver xenotransplantation. Therefore, there is enough evidence that porcine liver xenografts will provide an adequate function.

Infectious Disease Transmission

While potential infectious complications are well-characterized in *allo*transplantation and protocols have been developed to minimize transmission, in xenotransplantation, there exists a risk of zoonotic pathogen transmission from the non-human donor (i.e. pig) organ into the immunocompromised human host. In order to address this issue, exclusion lists have been generated to identify pathogenic organisms that could potentially be transmitted from swine to humans through xenotransplantation (52). Transmission of porcine endogenous retroviruses (PERV) has been researched extensively in the transplantation of pig tissues into NHPs, minks, rats, guinea pigs, and severe combined immunodeficiency (SCID) mice, however aside from a single case of transmission in a guinea pig there have been no other documented cases to date (53). Additionally, pig-derived pathogens have not been identified in immunosuppressed humans with the exception of hepatitis E virus (HEV) (2). Since it is essentially impossible to predict which organisms will pose a health hazard until clinical trials are conducted, the current approach to addressing the issue of infectious disease transmission in pig-to-human xenotransplantation is rigorous selection of "pathogen-free" pigs (2). This is achieved through herd isolation, continuous surveillance of source animals, meticulous breeding records, microbiological assessments, and standard veterinary care to ensure the overall health of donor pigs. Breeding swine in biosecure facilities will additionally minimize swine exposure to pathogens. The first genetically modified pig-to-human cardiac xenotransplantation took place on January 7, 2022, a ground-breaking advancement in the field of xenotransplantation. This landmark event will provide a rare and valuable opportunity to

observe and evaluate potential infectious complications in a true pig-to-human xenotransplant.

Patient Consent

There are many ethical considerations and regulatory aspects in xenotransplantation, particularly with respect to patient consent and autonomy. These issues were previously discussed in detail (54, 55). Briefly, in patients with chronic liver failure who retain capacity to make their own medical decisions the issue of patient consent is straightforward. For patients who arrive to the hospital unconscious in fulminant hepatic failure, the current standard is to place them on the waiting list for an emergent human liver allotransplant. The current ethical framework for treating life-threatening emergencies differs substantially from non-emergency situations, in that informed consent is not required in order to treat. In a scenario where the pig liver xenotransplantation is a reasonable alternative, the clinician would be required to choose between placing the patient on the waiting list or proceeding with liver xenotransplantation with an informed consent obtained from the family and/or hospital's ethical committee.

FUTURE DIRECTIONS

Despite the considerable success achieved in preclinical liver xenotransplantation trials with survival of almost one month, there still exists a significant gap between where the field is now and where it needs to be to begin the first clinical pig-to-human liver xenotransplantation trial. There are several potential areas for future research to overcome the major barriers of thrombocytopenia and coagulation dysregulation.

Although pharmaceutical approaches such as anti-GpIb and/or anti-GpIIb/IIIa antibodies have the potential to ameliorate thrombocytopenia, there always exists a risk of unintended adverse events with polypharmacy. Genetic engineering of pigs, therefore, is the preferred approach. Based on preliminary results (30), human vWF has shown potential to reduce platelet consumption. Additional genetic modifications aimed at minimizing platelet sequestration by porcine LSECs and KCs include ASGR1-KO and transgenic expression of human CD18 and/or CD40, respectively. Given the duality of the SIRPα/CD47 interaction, genetic modification of this axis would likely require the concomitant knock-in strategy to delete porcine SIRPα and CD47 and replace them with the human counterparts. This approach may prove technically challenging and threaten pig viability (13), thus further research is needed to evaluate this possibility.

Developing an effective approach to stabilizing coagulation dysregulation in pig-to-NHP liver xenotransplantation models is imperative in order to progress to pig-to-human clinical trials. Pharmaceutical support with coagulation factor replacement and co-stimulation blockade will likely continue to be necessary to achieve sufficient survival times, however, introducing genetic modifications may augment its efficacy. Given the implications of TF in liver xenotransplantation-induced coagulation dysregulation described previously, genetic modifications to porcine TF may prove valuable (43). Additionally, further optimization of transgenic hTM expression in organ source pig livers would provide valuable information regarding its utility. Indeed, novel approaches in genetic engineering of pig cells and testing capabilities of their phenotypes without making those genetically modified pigs each time for each genetic modification will reduce the time and effort to understand which genetic modification combinations would be optimal for preclinical/clinical trials (56, 57). With the strong data in preclinical trials in pig-to-NHP xenotransplantation trials as well as recent progress in 'first-in-man' genetically-engineered pig heart xenotransplantation into a patient, we would also strongly suggest that complement regulatory proteins, such as CD55 and CD46 as well as hTM and human EPCR expression would be helpful in extending the xenograft survival (13, 42, 58). Another future gene target amenable to manipulation would be the expression of human albumin in pigs since pig albumin production is significantly low compared to human albumin levels (59). Alterations to baseline physiologic levels of porcine albumin may alter the viability of the donor pig, and could avoid long-term low albumin-related issues (e.g. ascites) in the recipient. Thus, further research is necessary to explore this approach.

CONCLUSION

As genetic modification techniques continue to improve, the potential of liver xenotransplantation as a bridge to allotransplantation in fulminant liver failure patients becomes increasingly apparent (23, 60). Since genetically-engineered pigs with multiple gene knockouts and knock-ins have been generated (over ten genes) while maintaining donor viability, at present, there appears to be no fundamental limit to how much the porcine genome can be edited before the well-being of the donor pig is compromised. As such, the genetic modifications proposed here have the potential to greatly improve pig-to-NHP liver xenograft survival. Combined with tailored immunosuppression and coagulation factor support, the first pig-to-human liver xenotransplantation clinical trial may well be just over the horizon.

AUTHOR CONTRIBUTIONS

AC-N wrote the first draft under the supervision of BE. All authors contributed to the writing and critical review of the manuscript. All authors contributed to the article and approved the submitted version.

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Serum Antibody Binding and Cytotoxicity to Pig Cells in Chinese Subjects: Relevance to Clinical Renal Xenotransplantation

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Kidney xenotransplantation is expected to contribute to resolving the shortage of kidneys from deceased human donors. Although progress in experimental life-supporting pig renal xenotransplantation has been encouraging, there are still issues to be considered before a clinical trial can be initiated. We attempted to clarify some of these by an *in vitro* study. Blood was drawn from healthy volunteers (Volunteers, n=20), patients with end-stage renal disease (ESRD, n=20) pre-operation (Pre), and on Day 1 (POD 1) and Day 14 (POD 14) after renal allotransplantation, brain-dead organ donors (DBD, n=20), and renal allotransplant recipients who were currently experiencing T cell-mediated rejection (Allo-TCMR, n=20). Serum IgM/IgG binding to, and complement-dependent cytotoxicity (CDC) of, PBMCs and RBCs from (a) wild-type (WT), (b) α1,3-galactosyltransferase gene-knockout (GTKO), (c) GTKO/beta-1,4-N-acety1 galactosaminyltransferase 2-knockout (GTKO/β4GalNT2KO), (d) GTKO/cytidine monophosphate-N-acetylneuraminic acid hydroxylase-knockout (GTKO/CMAHKO), and (e) GTKO/β4GalNT2KO/CMAHKO/hCD55 (TKO/hCD55) pigs were measured by flow cytometry. We obtained the following results: (i) Serum IgM/IgG binding and CDC in Volunteers were significantly greater to WT, GTKO, and GTKO/β4GalNT2KO PBMCs or RBCs than to GTKO/CMAHKO and TKO/hCD55 cells; (ii) ESRD, DBD, and Allo-TCMR serum antibody binding and CDC to WT pig PBMCs were significantly greater than to GTKO, GTKO/β4GalNT2KO, GTKO/CMAHKO, and TKO/hCD55 cells; (iii) antibody binding to GTKO/CMAHKO pig cells was significantly lower in hemodialysis than peritoneal dialysis patients. (iv) Two of twenty allotransplantation recipients' serum IgG binding to GTKO pig PBMCs increased on POD14 compared with Pre, but IgG binding to GTKO pig RBCs did not; (v) In all sera, the lowest antibody binding and CDC were to GTKO/CMAHKO and TKO/CD55 pig cells. We conclude (i) CMAHKO in the pig may be critical to the success of clinical pig

kidney xenotransplantation, and may be the most important after GTKO, at least in Chinese patients; (ii) subjects with ESRD, or who are immunosuppressed after kidney allotransplantation, and DBD, have lower levels of antibody binding and CDC to genetically-engineered pig cells than do volunteers; (iii) TKO pigs with selected human 'protective' transgenes, e.g., CD55, are likely to prove to be the optimal sources of kidneys for clinical xenotransplantation.

Keywords: brain-dead organ donors, complement-mediated cytotoxicity, end-stage renal disease, kidney, pig, xenotransplantation

INTRODUCTION

There is a critical shortage of deceased human donor organs for transplantation in patients with end-stage renal disease (ESRD) (1). Genetically-engineered pigs are a potential alternative source of kidneys for these patients. Pig-to-nonhuman primate kidney transplantation Is now associated with encouraging results with recipient and graft survival extending to >1 year in several cases (2–4). However, there are still some major issues that must be resolved before a clinical trial can be initiated, e.g., (i) what genetically-modified pigs should be the sources of kidneys for clinical renal xenotransplantation; and (ii) whether a new xenotransplantation model needs to be identified because of differences in antibody binding and complement-dependent cytotoxicity (CDC) to pig cells between humans and Old World monkeys (OWMs) (5).

Pigs that do not express Gal or Sda (GTKO/β4GalNT2KO pigs), with or without added human transgenes, may be the optimal source of organs for OWMs (**Table 1**), whereas pigs in which expression of all 3 known carbohydrate xenoantigens has been deleted [triple-knockout (TKO) pigs], with or without added human transgenes, are likely to be optimal for human recipients (5–8). Humans have low (or no) antibody levels and CDC to cells from TKO pigs.

Whether or not swine leukocyte antigen (SLA) expression needs to be deleted remains uncertain. SLA is the homolog of human leukocyte antigen (HLA), a protein complex expressed on human tissue capable of stimulating the development of new antibodies in allotransplantation. Some *in vitro* studies have indicated that HLA-sensitized patients will not be at greater risk of rejecting a pig organ than HLA-non-sensitized patients (9–14), but other studies indicate that HLA-sensitized patients have a greater risk of rejecting a pig organ (15–17), and so it would be prudent not to select HLA-sensitized patients for the first clinical trials of pig kidney transplantation (18).

Subjects with brain-death (DBD subjects) are a frequent source of organs for transplantation, and transplantation of a pig organ into a brain-dead human *recipient* has recently been carried out (19). However, brain death is associated with dysfunction of the cardiovascular, pulmonary, endocrine, thermoregulation, renal, hematologic and inflammatory systems (20–23). If DBD subjects are used as *recipients* in preclinical models of pig renal xenotransplantation, there is concern that these pathophysiological consequences may affect the xenograft, e.g., by activation of T and B lymphocytes, release of cytokines, etc. (19).

The aims of the present study were (i) to measure serum anti-pig antibodies in healthy human volunteers, patients with ESRD pre- and post-renal allotransplantation, DBD subjects, and patients with renal allografts who were currently experiencing acute T cell-mediated rejection (Allo-TCMR), and (ii) to provide further data to help select pigs with the optimal genotype for clinical renal xenotransplantation.

MATERIALS AND METHODS

Human Sera

Blood was drawn from (i) healthy volunteers (Volunteers, n=20; ABO blood types A n=6; B n=6; AB n=3; O n=5), (ii) patients with ESRD (n=20), pre-renal transplantation (Pre) and on Day 1 (POD 1) and Day 14 (POD 14) after renal allotransplantation,

TABLE 1 | Sources of human sera and types of pig cells used in these studies.

Human sera tested (and abbreviations used)

1. Healthy volunteers (Volunteers)
2. Patients with end-stage renal disease (ESRD) pre-kidney allotransplantation (Pre), and post-kidney allotransplantation on day 1 (POD1) and on day 14 (POD14)
3. Brain-dead organ donors (DBD)
4. Patients with kidney allografts that were currently experiencing acute T cell-mediated rejection (Allo-TCMR).

Pig cells (PBMCs and RBCs) tested (and abbreviations used)

1. Wild-type (WT, i.e., genetically-*un*modified)
2. α1,3-galactosyltransferase gene-knockout (GTKO)
3. GTKO/β-1,4N-acetylgalactosaminyltransferase gene-knockout (GTKO/β4GalNT2KO)
4. GTKO/cytidine monophosphate-N-acetylneuraminic acid hydroxylase gene-knockout (GTKO/CMAHKO).
5. Triple knockout (i.e., GTKO/β4GalNT2KO/CMAHKO) + transgenic expression of the human complement-regulatory protein, CD55 (TKO/hCD55).

Abbreviations: Allo-TCMR, allograft recipients who were currently experiencing T cell-mediated rejection; β4GalNT2KO, beta-1,4-N-acety1 galactosaminyltransferase 2-knockout; CMAHKO, cytidine monophosphate-N-acetylneuraminic acid hydroxylase-knockout; CDC, complement-dependent cytotoxicity; DBD, brain-dead donors; ESRD, end-stage renal disease; GTKO, α1,3-galactosyltransferase-knockout; HLA, human leukocyte antigens; hCD55, human complement-regulatory protein, CD55; OWMs, Old World monkeys; PBMCs, peripheral blood mononuclear cells; RBCs, red blood cells; SLA, swine leukocyte antigens; WT, wild-type.

(iii) brain-dead organ donors (DBD, n=20) and (iv) patients with renal allografts who were currently experiencing episodes of acute T cell-mediated rejection (Allo-TCMR, n=20) (**Table 1**). Sera were obtained from de-identified remnant/discarded clinical laboratory samples.

Sera from Volunteers were obtained from the Second Affiliated Hospital of Hainan Medical University, and all experimental protocols were approved by the ethics committee of the Second Affiliated Hospital of Hainan Medical University. All procedures involving humans were performed in accordance with the relevant guidelines and regulations, and had no adverse effects on the subjects.

Pigs

Blood was obtained from wild-type (WT, i.e., genetically-*un*modified) pigs (n=4) and from different genetically-modified pigs (n=4) (**Table 1**) (Chengdu Clonorgan Biotechnology, Chengdu, Sichuan, China).

Detection of Expression of Xenoantigens on Selected Pig Cells by Flow Cytometry

Pig RBCs and PBMCs were stained for expression of Gal (by isolectin BSI-B4), Sda (Dolichos biflorus agglutinin,DBA), Neu5Gc (chicken anti-Neu5Gc mAb), and SLA (anti-human β2-microglobulin antibody, β2M).

Binding of Human Serum IgM and IgG to pRBCs and pPBMCs by Flow Cytometry

Binding of human antibodies to pig cells was measured by flow cytometry using the relative geometric mean (rGM), as previously described (11). Briefly, pRBCs were separated from whole blood, washed x3 with phosphate-buffered saline (PBS), and centrifuged at 700g for 5min at 4°C. The washed RBCs were suspended in fluorescence-activated cell sorting (FACS) buffer (PBS containing 1% bovine serum albumin). pPBMCs were isolated using Ficoll (HaoYang, Tianjin, China) and suspended in FACS buffer for IgM/IgG binding assays. The isolated pRBCs ($5x10^5$/tube) and pPBMCs ($5x10^5$/tube) were incubated with heat-inactivated human serum at 4°C for 30min, respectively, and the final serum concentration was 20%. After incubation, cells were washed with PBS to remove unbound antibodies and were blocked with 10% goat serum for 15min at 4°C. After further washing with PBS, anti-human IgM or anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) (IgG: concentration 1:1000 for pRBCs and pPBMCs; IgM: concentration 1:1600 for pRBCs and pPBMCs) was added, and the cells were incubated for 30min at 4°C. After washing with PBS, 100μL PBS buffer was added. Flow cytometry was carried out using BD FACSCelesta (Becton Dickinson, San Jose, CA, USA).

Human Serum CDC of Pig PBMCs by Flow Cytometry

Briefly, PBMCs ($5×10^5$ cells in 250μL FACS buffer) were incubated with 50μL heat-inactivated human serum at 4°C for 1h. After washing with PBS, FACS buffer (200μL) and rabbit complement (50μL, Cedarlane, Hornby, CA, USA) were added (final concentration 20%), and incubation was carried out at 37°C for 30min. After washing with PBS, the cells were incubated in the dark at 4°C for 15min with propidium iodide, and finally 200μL FACS buffer was added. Flow cytometry was carried out using BD FACSCelesta.

Cytotoxicity was calculated, as follows (11):

$$\% \text{ cytotoxicity} = ([A - C]/[B - C]) \times 100$$

where A represented the percentage of dead cells, B was the maximal percentage of dead cells (PBMCs fixed with 70% ethanol), and C was the minimal percentage of dead cells (PBMCs incubated with medium only).

Statistical Analysis

Significance of the difference between two groups was determined by student t-test or Wilcoxon test. Continuous variables were expressed as mean ± SD. Comparisons among multiple groups were performed using a One-way ANOVA test (Tukey test) or nonparametric test (Dunn's test). A p value of <0.05 was considered statistically significant. All statistical analyses were performed using social sciences software GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

RESULTS

Expression of Gal, Sda, Neu5Gc, and SLA on Pig PBMCs and/or RBCs

PBMCs

PBMCs from WT pigs expressed Gal, Sda, Neu5Gc, and SLA (**Figure 1A**). As anticipated, PBMCs from a GTKO pig did not express the Gal antigen, but expressed Sda and Neu5Gc. Those from a GTKO/β4GalNT2KO pig did not express Gal or Sda, but expressed Neu5Gc. Those from a GTKO/CMAHKO pig did not express Gal or Neu5Gc, but expressed Sda. And those from a TKO/hCD55 pig did not express any of the 3 carbohydrate xenoantigens, but expressed hCD55. Neu5Gc that was still expressed a small amount in GKTO/CMAHKO and TKO/hCD55 pig PBMCs was a false positive which was caused by Rabbit anti-Chicken IgY/Alexa Fluor 555 antibody (**Supplemental Material**).

There was a positive expression The false positive expression of Neu5Gc on GTKO/CMAHKO and TKO/hCD55 pig PBMCs were caused by Rabbit anti-Chicken IgY/Alexa Fluor 555 antibody (**Supplemental Material**).

RBCs

Expression of xenoantigens on RBCs from all of the above pigs followed the same pattern as that to PBMCs except that they did not express SLA or hCD55 (**Figure 1B**).

Effect of Different Human Sera on IgM and IgG Binding and CDC to WT and Various Genetically-Modified Pig PBMCs by Flow Cytometry

The aim was to compare the binding of different human sera to various pig PBMCs.

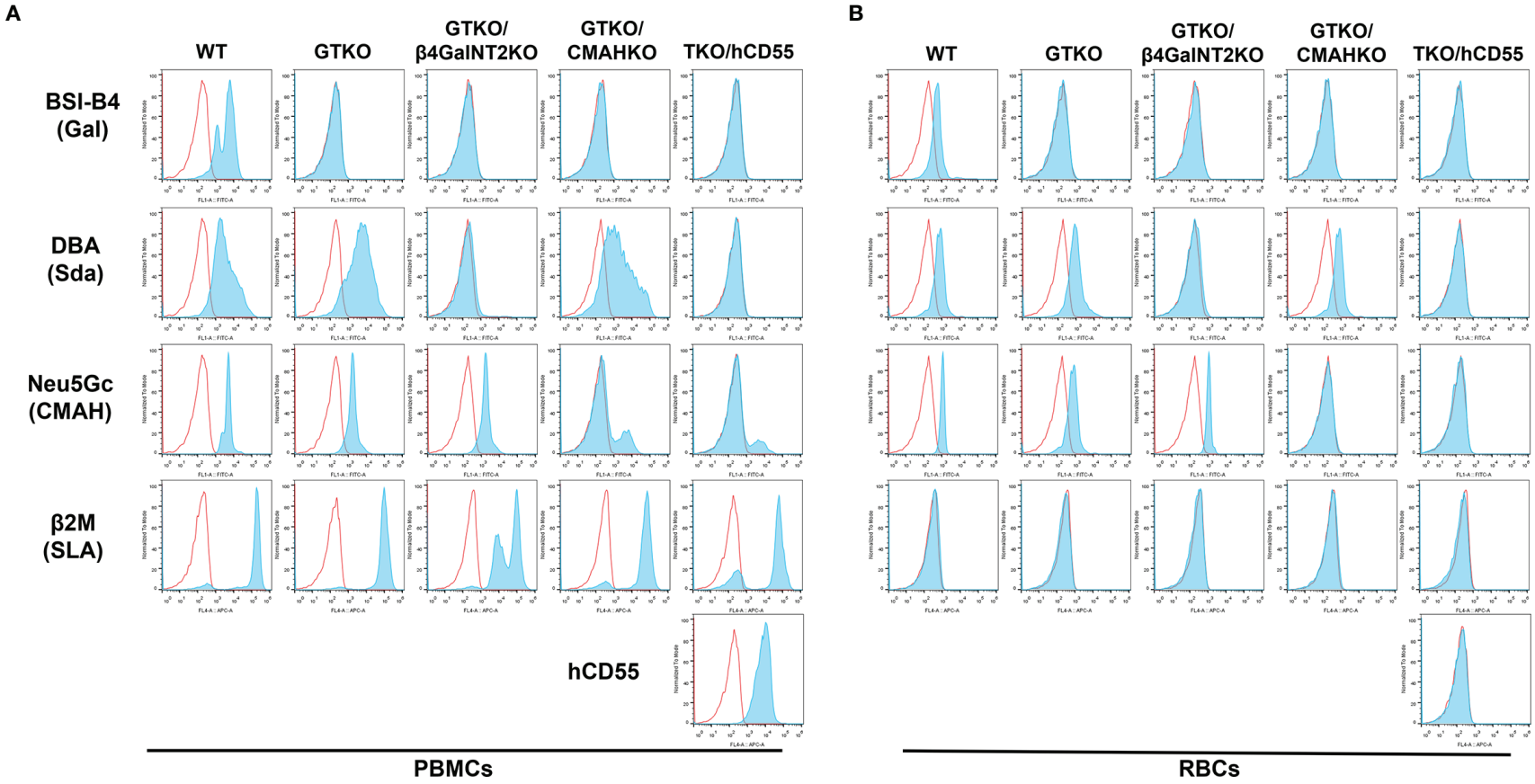

FIGURE 1 | Expression of Gal, Sda, Neu5Gc, SLA, and hCD55 on WT, GTKO, GTKO/β4GalNT2KO, GTKO/CMAHKO and TKO/hCD55 pig PBMCs and RBCs by flow cytometry. **(A)** PBMCs and **(B)** RBCs from WT pigs expressed Gal, Sda, and Neu5Gc. GTKO expressed Sda and Neu5Gc. GTKO/β4GalNT2KO expressed Neu5Gc. GTKO/CMAHKO expressed Sda. TKO/hCD55 PBMCs (but not RBCs) expressed hCD55. All PBMCs expressed SLA, but RBCs did not express SLA.

IgM Binding

Mean IgM binding to WT, GTKO and GTKO/β4GalNT2KO PBMCs was significantly greater in Volunteers than in the other three sera. Mean IgM binding to GTKO/CMAHKO and TKO/hCD55 PBMCs was minimal and not significantly different between all four groups of sera (Volunteers, ESRD, DBD, and Allo-TCMR) (**Figure 2A**).

IgG Binding

Mean IgG binding to WT, GTKO and GTKO/β4GalNT2KO PBMCs was significantly greater in Volunteers than in the other three sera. No sera showed more than minimal binding to GTKO/CMAHKO and TKO/CD55 PBMCs (**Figure 2B**).

CDC

Mean serum CDC to GTKO/CMAHKO and TKO/hCD55 pig PBMCs was not significantly different between Volunteers, ESRD, DBD, and Allo-TCMR (**Figure 2C**). However, mean CDC to WT, GTKO and GTKO/β4GalNT2KO pig PBMCs was significantly greater in Volunteers than in ESRD, DBD, and Allo-TCMR. CDC of GTKO/CMAHKO PBMCS was low or negative in all sera, and almost no sera caused any killing of TKO/CD55 PBMCs.

Effect of Different Human Sera on IgM and IgG Binding to WT and Various Genetically-Modified Pig RBCs by Flow Cytometry

The aim was to compare the binding of different human sera to various pig RBCs.

IgM Binding

Mean IgM binding to WT, GTKO and GTKO/β4GalNT2KO RBCs was significantly greater in Volunteers compared with ESRD, DBD, and Allo-TCMR. Mean IgM binding to GTKO/CMAHKO and TKO/hCD55 RBCs was minimal in all sera and

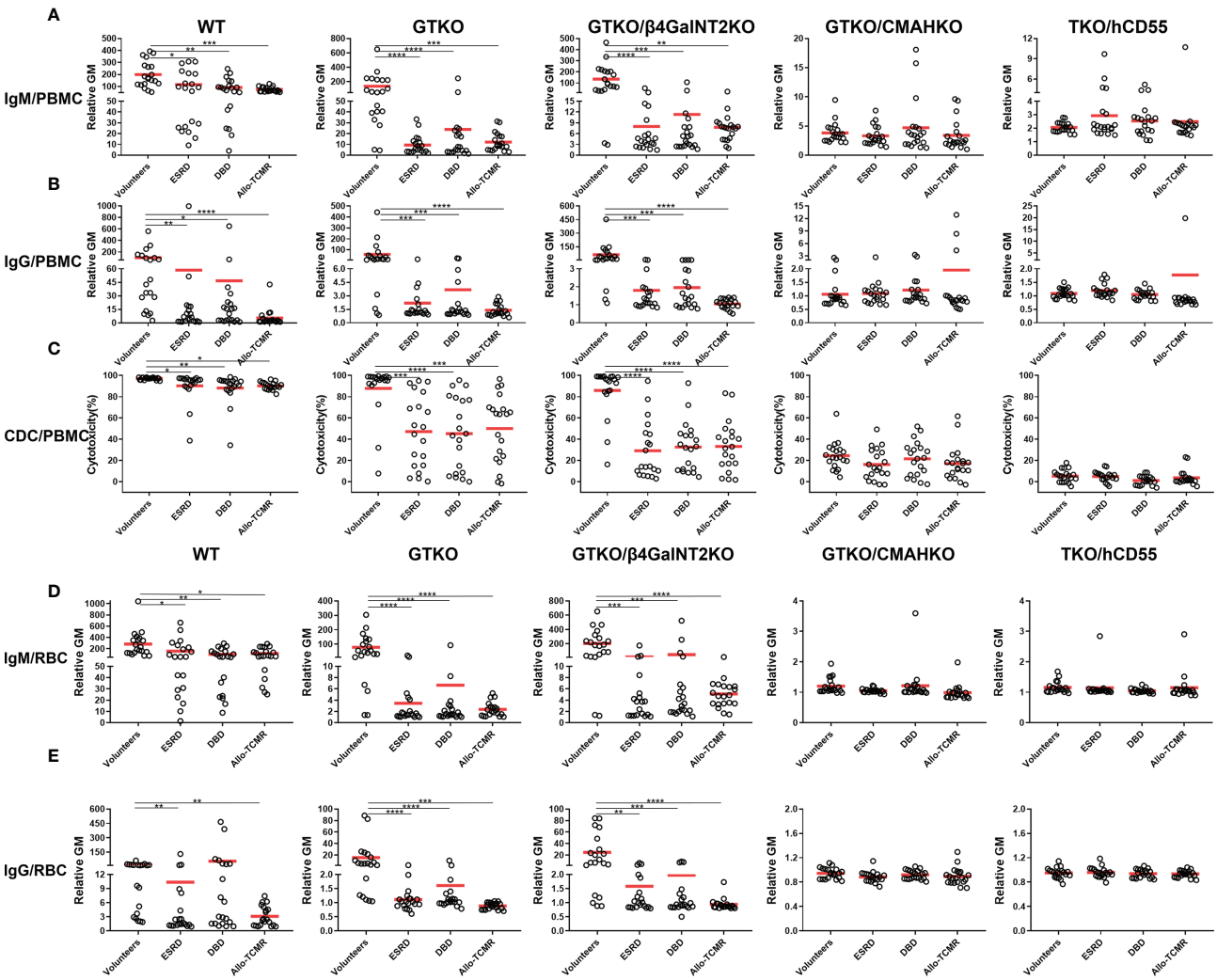

FIGURE 2 | Continued

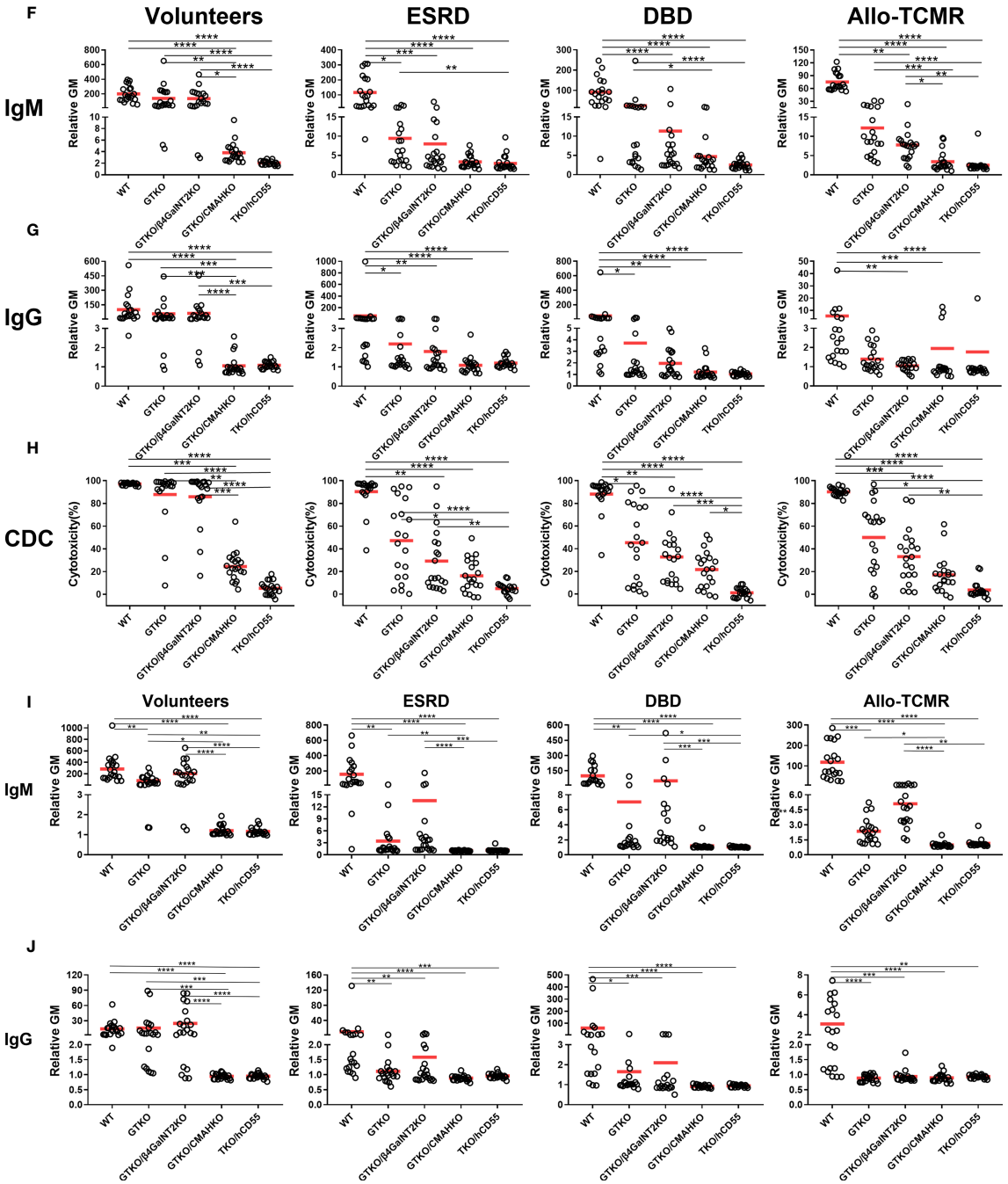

FIGURE 2 | Effect of different human sera on IgM and IgG binding and CDC to various pig PBMCs and RBCs and effect of genetic-engineering of pig PBMCs and RBCs on human IgM and IgG binding and CDC by flow cytometry. Comparison of mean **(A)** IgM/**(B)** IgG binding and **(C)** CDC of sera from Volunteers, ESRD, DBD and Allo-TCMR to WT, GTKO, GTKO/β4GalNT2KO, GTKO/CMAHKO, and TKO/hCD55 pig PBMCs. Mean of **(D)** IgM and **(E)** IgG comparing binding of sera from Volunteers, ESRD, DBD, and Allo-TCMR to WT, GTKO, GTKO/β4GalNT2KO, GTKO/CMAHKO, and TKO/hCD55 pig RBCs. (*p <0.05, **p<0.01, ***p<0.005, ****p<0.001). Volunteers had very low levels of IgM/IgG binding and CDC to GTKO/CMAHKO and TKO/hCD55 pig PBMCs **(F-H)** or RBCs **(I, J)**. IgM and IgG binding and CDC to GTKO, GTKO/β4GalNT2KO, GTKO/CMAHKO, and TKO/hCD55 PBMCs **(F–H)** or RBCs **(I, J)** were low in sera from ESRD, DBD, and Allo-TCMR. (*p<0.05, **p<0.01, ***p<0.005, ****p<0.001).

was not significantly different between all four groups of sera (**Figure 2D**).

IgG Binding

Mean IgG binding to WT was significantly greater in Volunteers compared with ESRD an Allo-TCMR. Mean IgG binding to GTKO and GTKO/β4GalNT2KO RBCs was significantly greater in Volunteers than in ESRD, DBD, and Allo-TCMR. Mean IgG binding was absent or minimal to GTKO/CMAHKO and TKO/CD55 RBCs (**Figure 2E**).

The Effect of Genetic-Engineering of Pig PBMCs on Human IgM and IgG Binding and CDC by Flow Cytometry

The data presented in relation to differences in binding of various human sera to pig cells (**Figures 2A–E**) were re-presented to more clearly illustrate the effect of different pig genotypes (**Figures 2F–J**).

IgM Binding

Mean IgM binding in serum from Volunteers to WT, GTKO, and GTKO/β4GalNT2KO PBMCs was significantly greater than to GTKO/CMAHKO and TKO/hCD55 PBMCs. Mean IgM binding in serum from ESRD to WT PBMCs was significantly greater than to GTKO, GTKO/β4GalNT2KO, GTKO/CMAHKO and TKO/hCD55 PBMCs, and mean IgM binding to GTKO PBMCs was significantly greater than to TKO/hCD55 PBMCs. Mean IgM binding in serum from DBD and Allo-TCMR to WT PBMCs was significantly greater than to GTKO/β4GalNT2KO, GTKO/CMAHKO and TKO/hCD55 PBMCs, and the mean IgM binding to GTKO PBMCs was significantly greater than to GTKO/CMAHKO and TKO/hCD55 PBMCs, what's more, Mean IgM binding in serum from Allo-TCMR to GTKO/β4GalNT2KO PBMCs was significantly greater than to GTKO/CMAHKO and TKO/hCD55 PBMCs (**Figure 2F**).

In healthy human sera (Volunteers), mean IgM binding was reduced by approximately 20% by GTKO (compared to WT), but by approximately 80% by CMAHKO (WT vs GTKO: 199 vs 138, p=ns; WT vs CMAHKO: 199 vs 4, p<0.001) (**Figure 2F**).

IgG Binding

Mean IgG binding was almost lower than mean IgM binding in all sera (**Figure 3A**). Mean IgG binding in Volunteers to WT, GTKO and GTKO/β4GalNT2KO PBMCs was significantly greater than to GTKO/CMAHKO and TKO/hCD55 PBMCs. Mean IgG binding in ESRD and DBD to WT PBMCs was significantly greater than to GTKO, GTKO/β4GalNT2KO, GTKO/CMAHKO and TKO/hCD55 PBMCs. Mean IgG binding in serum from Allo-TCMR to WT PBMCs was significantly greater than to GTKO/β4GalNT2KO, GTKO/CMAHKO and TKO/hCD55 PBMCs (**Figure 2G**). In all sera, binding to GTKO/CMAHKO and TKO/CD55 PBMCs was minimal or absent.

In healthy human sera (Volunteers), mean IgG binding was reduced by approximately 5% by GTKO (compared to WT) but by approximately 90% by CMAHKO (WT vs GTKO: 100 vs 58, p=ns; WT vs CMAHKO: 100 vs 1, p<0.01)

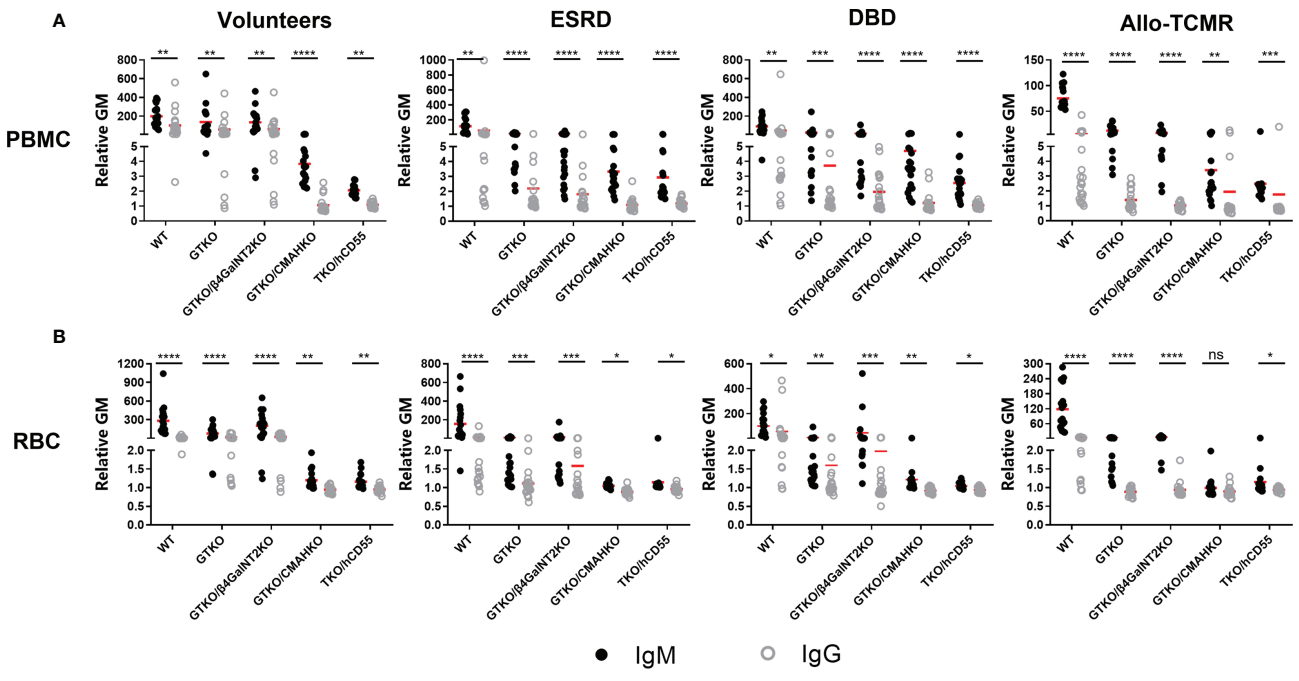

FIGURE 3 | Comparison of serum IgM and IgG binding to PBMCs and RBCs. All human subjects had lower levels of IgG binding (compared to IgM) to WT, GTKO, GTKO/β4GalNT2KO, GTKO/CMAHKO, and TKO/hCD55 PBMCs **(A)** and RBCS **(B)**. (*p<0.05, **p<0.01, ***p<0.005, ****p<0.001).

CDC

Although there were many variations in CDC depending on the serum and the source of PBMCs (**Figure 2H**), the most obvious finding was that CDC to GTKO/CMAHKO and, particularly, TKO/CD55 PBMCs was generally low or absent.

In healthy human sera (Volunteers), mean CDC was reduced by approximately 10% by GTKO (compared to WT) but by approximately 80% by CMAHKO (WT vs GTKO: 97 vs 88, p=ns; WT vs CMAHKO: 97 vs 25, p<0.001).

The Effect of Genetic-Engineering of Pig RBCs on Human IgM and IgG Binding by Flow Cytometry

IgM Binding

Mean serum IgM binding followed the same pattern as for IgM binding to PBMCs, with minimal binding to GTKO/CMAHKO and TKO/CD55 RBCs in all sera (**Figure 2I**).

IgG Binding

Mean serum IgG binding was lower in all sera than IgM binding (**Figure 3B**). Binding was greatest in the sera from Volunteers, but again was minimal to GTKO/CMAHKO and TKO/CD55 RBCs (**Figure 2J**).

Comparison of IgM/IgG Antibody Binding and CDC to Pig PBMCs or RBCs Between Hemodialysis Patients and Peritoneal Dialysis Patients

Patients with ESRD were divided into 2 groups based on whether they were undergoing hemodialysis (n=15) or peritoneal dialysis (n=5).

IgM/IgG Binding

There were no significant differences in IgM/IgG binding to WT, GTKO, GTKO/β4GalNT2KO, and TKO/hCD55 PBMCs and RBCs between hemodialysis and peritoneal dialysis patients (**Figure 4**), but serum IgM binding to GTKO/CMAHKO PBMCs and RBCs was significantly lower in hemodialysis patients than in peritoneal dialysis patients.

CDC

There was no significant difference in CDC to any cell type between hemodialysis and peritoneal dialysis patients (**Figure 4**).

Changes in the Levels of IgM and IgG Binding in Patients With ESRD Who Underwent Kidney Allotransplantation

Serum samples from renal allotransplant recipients were collected pretransplant (Pre), and on POD1 and POD14, and the levels of anti-pig IgM and IgG antibodies were measured (**Figure 5**). In the majority of patients (n=18), there was no change in IgM/IgG binding to PBMCs or RBCs between Pre, POD 1, and POD 14, although in some patients anti-pig IgM/IgG decreased transiently on POD1, but recovered to Pre levels by POD14. (This may possibly be related to hemodilution by perioperative fluid infusion.) However, in 2 of the recipients (red dot), serum IgG binding to GTKO PBMCs increased by POD14, compared with Pre and POD1. There was no change in IgG binding to GTKO RBCs (that do not express SLA), nor in binding to PBMCs and RBCs from the other pig genotypes.

Influence of ABO Blood Type of Healthy Human Volunteers on Serum IgM and IgG Binding and CDC to WT and Various Genetically-Modified Pig PBMCs or RBCs by Flow Cytometry

Healthy volunteers were divided into 4 groups based on ABO blood type.

IgM/IgG Binding

There were no significant differences in IgM/IgG binding to WT, GTKO, GTKO/β4GalNT2KO, and TKO/hCD55 PBMCs and RBCs in relation to ABO blood type, but IgM binding to GTKO/CMAHKO RBCs was significantly lower in sera from subjects with A blood type compared to those with O blood type (**Figure 6**).

CDC

There was no significant difference in serum CDC between subjects of the four blood types (**Figure 6**).

DISCUSSION

Effect of CMAHKO

The first important observation in this study was that our data showed that healthy volunteer serum IgM binding to WT, GTKO, GTKO/β4GalNT2KO PBMCs was significantly greater than to CMAHKO and TKO/hCD55 PBMCs, strongly suggesting that CMAHKO will be important for clinical renal xenotransplantation, as others have also reported (24, 25). However, deletion of expression of Neu5Gc appeared to play a more important role in reducing human antibody binding to the pig cells than deletion of expression of Gal, which is in contrast to some previous studies in which GTKO had a much greater impact than CMAHKO on IgM binding (14, 26). The reduction in IgM and IgG binding to GTKO pig cells when compared to WT cells was less than reported in most previous studies (25), but, in contrast, the reduction in binding and CDC was significantly greater after deletion of expression of Neu5Gc. This is most likely explained by differences in the antibody profiles of the Chinese participants in this study compared with those of some other ethnic groups.

Neu5Gc is expressed in pigs, apes and OWMs, but not in humans (27–29), and therefore only humans develop anti-Neu5Gc antibodies (30). Gao and her colleagues drew attention to the fact that natural antibodies are largely associated with exposure to glycans expressed on flora in the gastrointestinal tract (26), as suggested by others previously (31). In humans, anti-Neu5Gc antibodies develop during the first 6 months of life, and reach adult levels by the end of the first year (32). Both anti-Neu5Gc IgM and IgG increase soon after the

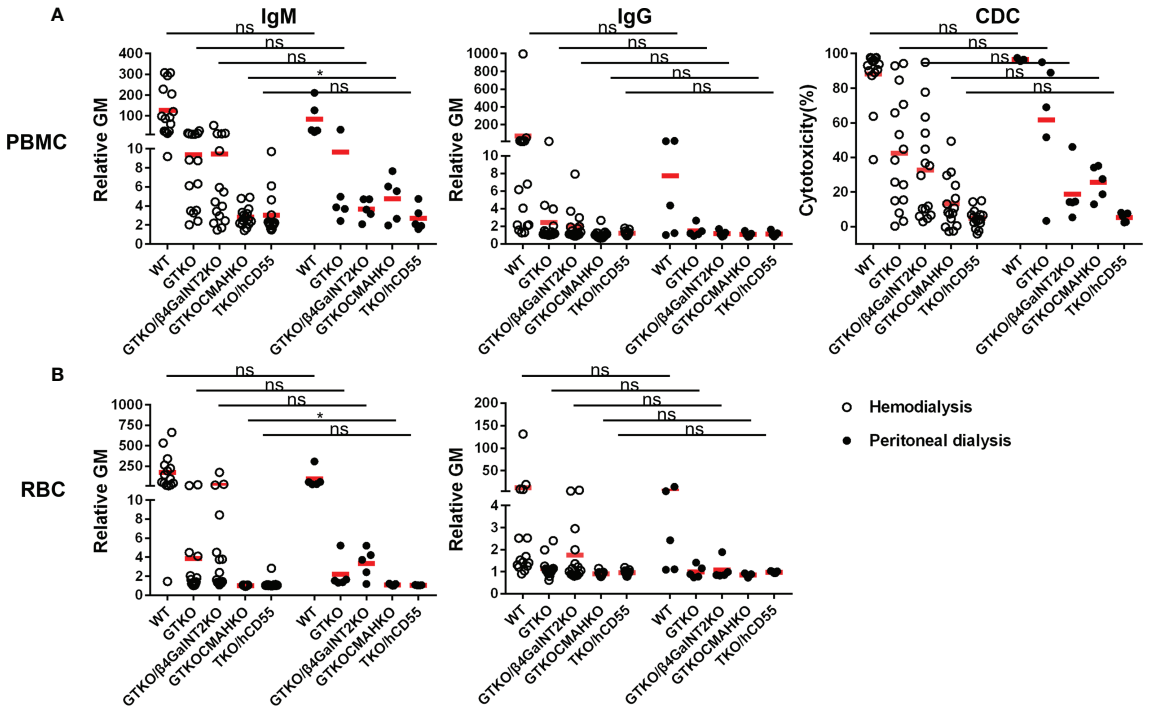

FIGURE 4 | Comparison of serum IgM/IgG binding and CDC to PBMCs or RBCs from WT, GTKO, GTKO/β4GalNT2KO, GTKO/CMAHKO and TKO/hCD55 pig in patients receiving hemodialysis or peritoneal dialysis. **(A)** There were no differences in IgM/IgG binding or CDC to WT, GTKO, GTKO/β4GalNT2KO, and TKO/hCD55 PBMCs, but IgM binding to GTKO/CMAHKO to PBMCs was significantly lower in hemodialysis patients. **(B)** There were no differences in IgM/IgG binding to WT, GTKO, GTKO/β4GalNT2KO, GTKO/CMAHKO, and TKO/hCD55 RBCs, but IgM binding to GTKO/CMAHKO to RBCs was again significantly lower in hemodialysis patients. (*p<0.05; ns=not significant).

infant is exposed to cow's milk and baby foods containing red meat, which express Neu5Gc (32).

All of the subjects from whom blood was drawn in the present study were Chinese and had been resident in China throughout most of their lives, whereas those in other reported studies were from a variety of ethnic and geographic backgrounds. This suggests that maybe in Chinese patients (or in patients who have been exposed to Chinese environmental factors, e.g., diet, for a prolonged period of time), an absence of expression of Neu5Gc in the pig organ may be as important, if not more important, than absence of expression of Gal.

We therefore suggest that the Chinese subjects included in the present study expressed different gastrointestinal flora (perhaps based on differences in diet), than other groups that have been studied in other geographic regions (26, 33), thus rendering Neu5Gc a more important stimulus to natural antibody production. However, of interest, no correlation between diet and anti-pig antibody levels was found in a previous study in Taipei (34). There may other 'ethnic' or 'environmental' differences in other population groups that have not yet been investigated.

There are increasing *in vitro* data indicating that TKO pig organs will prove to be a major advance over GTKO organs for transplantation into humans (5, 7, 35) which is consistent with our conclusions from the present study.

Healthy Human Volunteers vs Other Groups

A second major observation made in this study was that (i) patients with ESRD, brain-dead donors, and immunosuppressed patients with kidney allografts generally had significantly lower levels of anti-pig antibodies than healthy human volunteers, except in regard to WT pig cells. The trends in CDC were similar to those of IgM and IgG. In addition, the ABO blood type of the donor of the serum appeared to play no part in influencing the results.

The clinical impact of ESRD on anti-pig immunity remains to some extent uncertain because immune dysfunction in ESRD includes both immunoactivation and immunosuppression. Heparin-induced antibodies (HIA) (36, 37) and anticardiolipin antibodies (IgG-ACA) (38) are elevated in ESRD and the complement system can be activated (39, 40). However, It is well-recognized that ESRD patients are to some extent immunocompromised (41, 42), e.g., reduced number of NK cells, reduced phagocytic activity of neutrophils (43). Anti-pig antibodies are low in infants (44) and in patients with ESRD (14), the last of which observations is consistent with our present results.

Patients with kidney allografts are receiving chronic immunosuppressive therapy, and presumably this has prevented an increase in antibody levels even though T cell

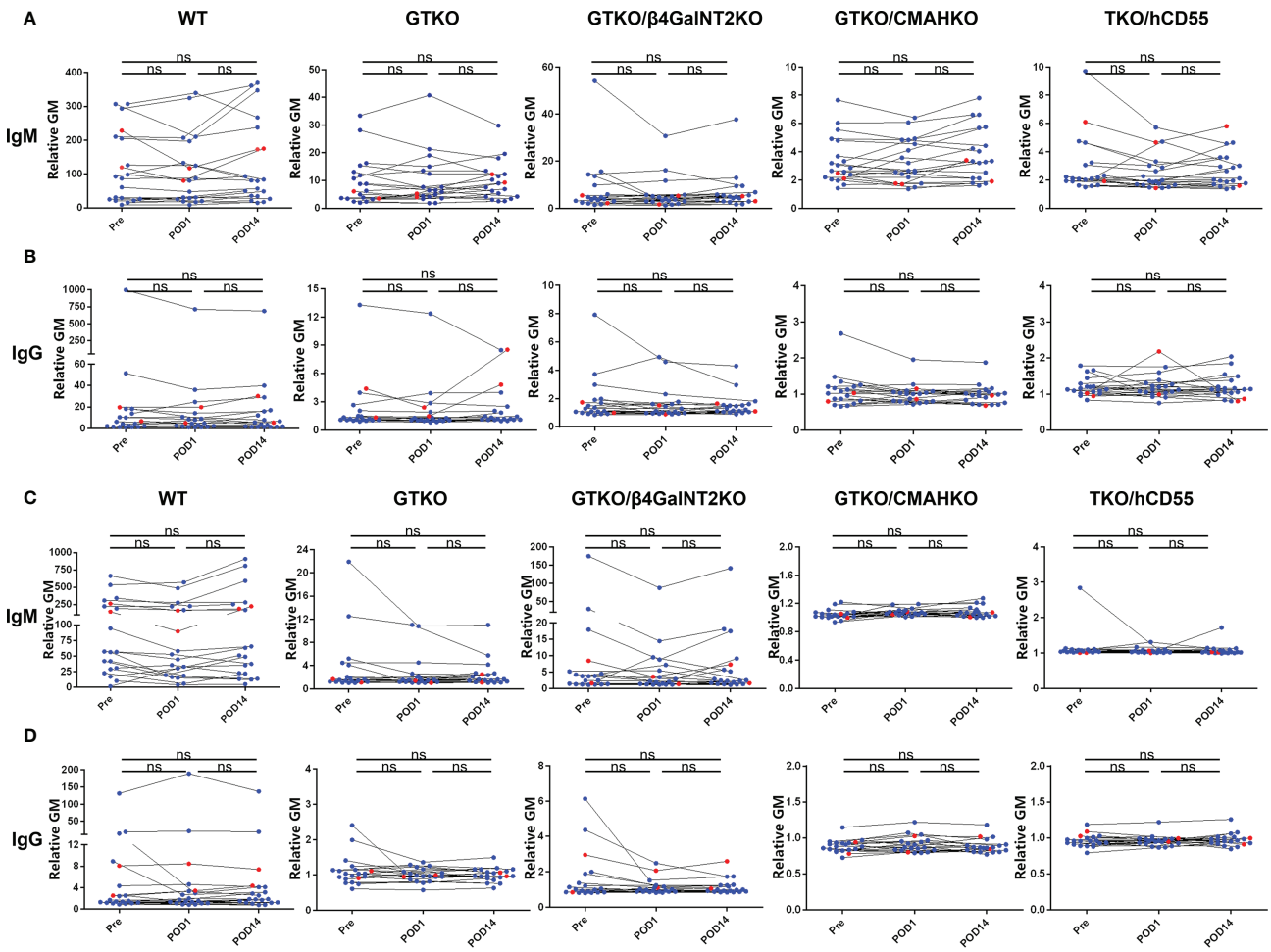

FIGURE 5 | Changes in IgM and IgG binding to pig cells in patients with ESRD pre and early post-renal allotransplantation. Serum samples from renal allotransplant recipients were collected Pre, and on POD1 and POD14 to measure IgM **(A)** and IgG **(B)** binding to pig PBMCs or to pig RBCs **(C, D)**. In the majority of cases, there were no significant changes in binding between the time-intervals, although in some there was a transient reduction in IgM and IgG binding on POD1 (possibly associated with hemodilution or binding to the pig cells) that had recovered to Pre levels by POD14. In two sera, IgG binding to GTKO PBMCs increased on POD14 compared with Pre and POD1, but there was no change in binding to GTKO RBCs, nor to pig cells of other genotypes. (ns, not significant).

activation had taken place in the patients we studied. The explanation in brain-dead donors is not so obvious, but may be associated with the infusion of fluids to maintain an adequate hemodynamic state, resulting in hemodilution.

However, there was no difference in serum IgM/IgG binding to GTKO/CMAHKO and TKO/hCD55 PBMCs or RBCs in the 4 groups, indicating that the effect of deletion of Neu5Gc expression on the pig cell was sufficient to reduce antibody binding and CDC to negligible levels whatever the source of the serum. Expression of CD55 on TKO pig PBMCs further reduced CDC of the cells.

Hemodialysis vs Peritoneal Dialysis

Our data showed that there was no significant difference in serum IgM/IgG binding to any of the pig cell types between hemodialysis and peritoneal dialysis patients, with the exception of lower IgM binding to GTKO/CMAHKO cells in patients on hemodialysis. This suggests that hemodialysis might remove anti-β4GalNT2 antibodies. However, it is unlikely that hemodialysis directly removes antibodies since standard hemodialysis filters typically have a cut-off size of between 10–20 kDa, whereas IgM/IgG molecules are >150 kDa, and are thus *not* removed by hemodialysis (45).

Experimental Models of Pig Organ Xenotransplantation

As is well-known, the most widely used xenotransplantation model is the genetically-modified pig-to-OWM. A major difference between humans and OWMs is that OWMs express Neu5Gc on the vascular endothelium, whereas humans do not. OWMs, therefore, are far from ideal models for xenotransplantation, as there is markedly increased OWM serum antibody binding and CDC to TKO pig cells. Although New World monkeys have some advantage in this respect (5), their small size and the

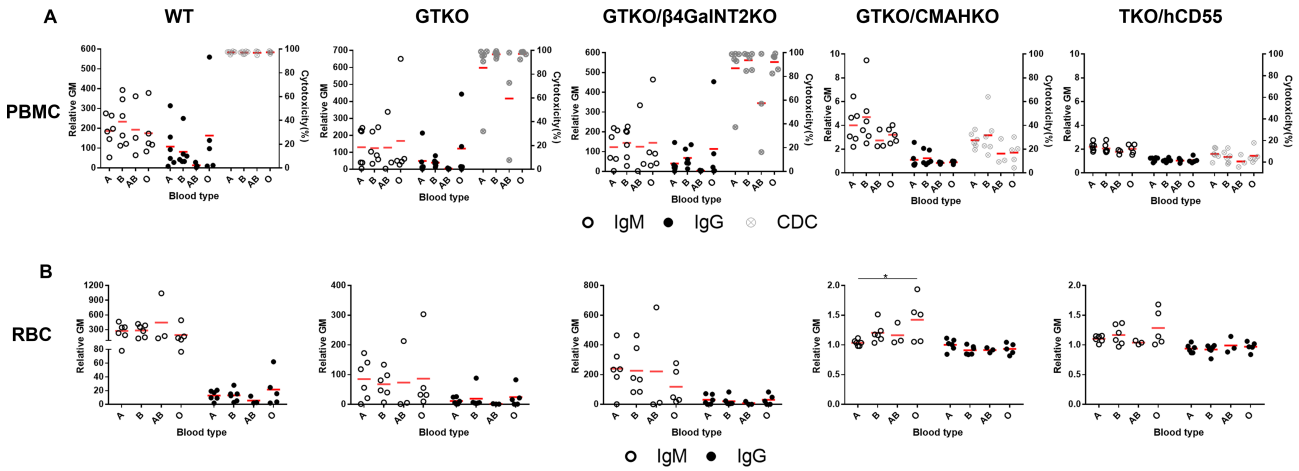

FIGURE 6 | Comparison of IgM/IgG binding and CDC to PBMCs or RBCs from WT, GTKO, GTKO/β4GalNT2KO, GTKO/CMAHKO and TKO/hCD55 pigs in sera from healthy human volunteers of different ABO blood types. **(A)** There were no differences in IgM (left) or IgG (middle) binding or CDC (right) to WT, GTKO, GTKO/β4GalNT2KO, GTKO/CMAHKO, and TKO/hCD55 PBMCs in sera from humans of various ABO blood types. **(B)** There were no differences in IgM (left) or IgG (right) binding to WT, GTKO, GTKO/β4GalNT2KO, and TKO/hCD55 RBCs, but IgM binding to GTKO/CMAHKO RBCs was significantly lower in sera from A blood type subjects than in those of O blood type. (*$p<0.05$).

ineffectiveness of some immunosuppressive drugs in them negates their suitability as a surrogate recipient for pig organ transplantation (46–50). Is there an alternative recipient as a surrogate for living humans?

Could DBD subjects be used as *recipients* in preclinical studies of xenotransplantation? Our data indicate that their levels of anti-pig antibodies are significantly lower than healthy volunteers (possibly associated with hemodilution through the need for fluid administration to maintain hemodynamic stability), and comparable to those in patients with ESRD. However, activation of innate immunity and inflammation can occur in brain-dead subjects (21). This observation, and because of their hemodynamic instability that may limit follow-up to days rather than months, reduces their suitability as potential surrogates for living recipients (51, 52).

Our observation that 2 of 20 patients undergoing kidney allotransplantation developed increased anti-pig antibody binding on POD14 may suggest that, at least in some patients, there may be cross-reactivity between anti-HLA antibodies and SLA epitopes, but the data are too few to draw any definite conclusions. (Unfortunately, anti-HLA antibodies were not investigated.)

Conclusions

In summary, on the basis of the present study, (i) CMAHKO in the pig may be critical to the success of clinical pig kidney xenotransplantation, and may be the most important after α1,3-galactosyltransferase gene be knockout, at least in Chinese patients; (ii) subjects with ESRD, or who are immunosuppressed after kidney allotransplantation, and brain-dead organ donors, all have lower levels of antibody binding and CDC to genetically-engineered pig cells than do healthy human volunteers; (iii) brain-dead subjects may mimic ESRD patients in that they both have low levels of anti-pig antibody levels, but experimental pig organ transplants in this group are unlikely to provide significant information of real value; (iv) TKO pigs with selected human 'protective' transgenes, e.g., CD55, are likely to prove to be optimal sources of kidneys for clinical xenotransplantation. (v) The role of SLAKO or SLA knockdown remains uncertain.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Second Affiliated Hospital of Hainan Medical University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by ethics committee of the Hainan Medical University.

AUTHOR CONTRIBUTIONS

YW and GC designed the experiments. TL, HF, JD, QX, and SH participated in the performance of the research and data analysis. DP provided transgenic pigs for experiments. TL, DC, and HJ prepared the figures and wrote the article. DC, YW, and GC

critically revised the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.844632/full#supplementary-material

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REVIEW
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The Influence of Microenvironment on Survival of Intraportal Transplanted Islets

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Clinical islet transplantation has the potential to cure type 1 diabetes. Despite recent therapeutic success, it is still uncommon because transplanted islets are damaged by multiple challenges, including instant blood mediated inflammatory reaction (IBMIR), inflammatory cytokines, hypoxia/reperfusion injury, and immune rejection. The transplantation microenvironment plays a vital role especially in intraportal islet transplantation. The identification and targeting of pathways that function as "master regulators" during deleterious inflammatory events after transplantation, and the induction of immune tolerance, are necessary to improve the survival of transplanted islets. In this article, we attempt to provide an overview of the influence of microenvironment on the survival of transplanted islets, as well as possible therapeutic targets.

Keywords: islet transplantation, microenvironment, instant blood, mediated inflammatory reaction, inflammatory cytokine, therapeutic target

INTRODUCTION

Although type 1 diabetes cannot be prevented or reversed, islet transplantation—which restores insulin independence and prevents severe hypoglycemia—is currently proving to be a promising treatment (1, 2). However, the results of multiple clinical trials have shown that most transplant recipients fail to achieve complete insulin independence. Although the immunosuppressive regimen reported from Edmonton, Canada, has achieved unprecedented success in achieving insulin independence in islet transplantation (3), there are still some problems that affect the outcome of islet transplantation. Advancements in the acquisition of pancreatic islets, immunosuppression of islet recipients, and an increase in the number of transplanted islets are required (4). The average human pancreas has 300,000 to 1.5 million pancreatic islets, and only 60% of this islet cell mass is needed to maintain a normal glucose metabolism (5). However, 72% of islet recipients still require more than two successfully processed islet preparations to obtain a sufficient beta cell mass to compensate for islet death in the post-transplantation period (6). However, the source of pancreatic islets is limited.

It was observed that only 10% of recipients remain insulin dependent for more than 5 years and that most recipients re-use insulin because of a decline in the islet function over time (7). During

transplantation, islet grafts are damaged by multiple challenges, including enzyme and mechanical damage caused by the isolation process, hypoxia, inflammation, immune rejection and toxicity of immunosuppressive drugs (8). The liver is currently the preferred transplantation site because the procedure is minimally invasive, easy to perform, and has low rates of bleeding and thrombosis (9). Early inflammatory responses strongly influence islet engraftment and survival after intrahepatic transplantation. This early immune response is triggered by immediate blood-mediated inflammatory response (IBMIR) and ischemia-reperfusion injury (10). Elevated inflammatory cytokines interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ), were observed during islet transplantation, and macrophages also involved in regulating the cell injury of transplanted pancreatic islets (11). It was observed that concomitant transplantation of islets and vascular endothelial cells in diabetic rats can prolong the survival of islet grafts (12). Previous studies have found that cytokine inhibitors or drugs that inhibit the activation of liver macrophage activation can improve the function of pancreatic islets after transplantation (13, 14). Changes in the microenvironment, mainly involving inflammatory cytokines, endothelial cells, and immune cells, play a key role in the survival of islet grafts.

This review discusses the changes in the microenvironment (inflammatory cytokines, immune cells, endothelial cells, etc.) during clinical intraportal islet transplantation, and their influence on the survival of the islet grafts, with the aim of improving the success of clinical islet transplantation

STATUS AND RECENT PROGRESS OF CLINICAL INTRAPORTAL ISLET TRANSPLANTATION

In 1990, David Scharp et al. reported the first case of type 1 diabetes allogeneic intraportal islet transplantation resulting in a short-term insulin independence, which opened the prelude to clinical islet transplantation (15). The International Islet Registry collected data from 267 islet allogeneic transplants from multiple centers between 1990 and 2001, only 12.4% of cases achieved insulin independence periods of at least 1 week, and 8.2% maintained insulin independence for more than 1 year. Appropriate immunosuppression remained a critical piece of the unsolved puzzle in order to improve long-term graft function and sustained insulin independence (16). Until 2000, Edmonton's group reported that insulin independence was achieved in seven patients with type 1 diabetes who underwent islet transplantation. This protocol established the need for sufficient islet β cell mass for transplantation and also proposed a glucocorticoid-free immunosuppressive regimen (3). Since the publication of the Edmonton protocol, many countries around the world have successively carried out allogeneic islet transplantation for the treatment of type 1 diabetes. Extended follow-up of these trials sho wed a gradual loss of insulin independence over time with renewed need for exogenous insulin, with only 10% of patients showing insulin independence at 5 years after transplantation (7). Hering et al. reported in 2016 that the North American Clinical Islet Transplant Consortium conducted a multicenter, single-arm, phase 3 trial to further evaluate the efficiency of allogeneic islet transplantation. The assay, using product purified human pancreatic islets, achieved an HbA1c of 5.6% in the absence of severe hypoglycemia (17). A subsequent multicenter, open-label, randomized controlled trial in Europe reconfirmed the benefit of islet transplantation over intensive insulin therapy in patients with type 1 diabetes with severe hypoglycemia or after kidney transplantation (18). Recently, Marco et al. proposed that vitamin D alone or in combination with other anti-inflammatory agents may serve as a new immunomodulatory strategy to reduce the recurrence of autoimmune/allogeneic transplant rejection in intraportal islet transplantation, but its safety and efficacy further validation in large prospective studies is required (19). In intraportal islet transplantation model, islet pretreatment with mitomycin C prolonged graft survival by suppressing pro-inflammatory events and inducing latent regulatory lymphocytes (20). Xenogeneic and stem cell-derived islet tissues have entered early clinical trials, although much remains to be learned about the *in vivo* physiology and immunogenicity of various products. These advances provide more options for cellular therapy in diabetes treatment, providing an unlimited source of pancreatic islet tissue for future applications (21).

INTRAPORTAL ISLET TRANSPLANTATION CHALLENGES AND STRATEGIES

Instant blood mediated inflammatory reaction (IBMIR)

The IBMIR, which is triggered by exposed tissue factor on the islet surface and characterized by platelet activation and aggregation, and activation of the coagulation and complement systems, is a major obstacle after islet transplantation. Moreover, it increases the infiltration of neutrophils, monocytes and macrophages (13). These reactions are thought to cause the immediate loss of most of the transplanted islets (22), and may also increase the risk of islet rejection later through adaptive immune responses (23). Potential means for reducing islet inflammation and protecting islets can be achieved by the addition of anti-inflammatory agents, anticoagulants or coating islets with various protective macromolecules during islet culture (24). *In vitro* and *in vivo* experiments demonstrated that low molecular weight dextran sulfate prevented IBMIR, leukocyte infiltration was eliminated at high doses, and transplanted pig islets survived significantly longer in recipients treated with low molecular weight dextran sulfate (25). Another phase II, multicenter, active-controlled, randomized study, the Clinical Islet Transplant Consortium 01 study, revealed that systemic low molecular weight dextran sulfate treatment showed similar efficacy to heparin treatment in preventing IBMIR and promoting islet engraftment (26). However, the reason for the current reluctance to use it is that its target- activated partial

thromboplastin time is 3 times longer than heparin's (27). The use of biotin/avidin technology to bind preformed heparin complexes to the islet surface resulted in protection against IBMIR *in vitro* loop model and in an allogeneic porcine model of clinical islet transplantation (28). *In vitro* and *in vivo* data suggested that activated protein C exerted anti-inflammatory and anti-apoptotic activities by directly acting on cells after exposure of hepatic endothelial cells to pancreatic islets (29). However, the above remain to be verified by clinical studies (30, 31). And it has been confirmed that Cibinetide can reduce IBMIR related platelet consumption in a pro-inflammatory environment and protect isolated human islets (32). The evidence showed that α-1 antitrypsin inhibited IBMIR, which resulted in improved outcome of intraportal islet transplantation in mouse model (33). Currently, the addition of heparin is the standard approach but it is insufficient (27).

COMPLEMENT SYSTEM

Complement activation is triggered by natural immunoglobulin (Ig)G or IgM. When isolated islets are exposed to blood, the complement system is rapidly activated and causes lysis of islet cells (34). Complement activation occurs *via* the classical pathway and an alternative pathway, which leads to the formation of a complex composed of C5b-9, which forms a "channel" through the cell membrane, leading to cell lysis and death. Another major function of the complement system is the production of anaphylatoxins C3a and C5a, which enhance the inflammatory response to islets (23). C3a and C5a produced by complement activation are powerful chemoattractants for macrophages and neutrophils (10). Activation through the C5a receptor can cause granulocytes to release enzymes, such as myeloperoxidase and elastase, and promote monocytes to release cytokines, such as IL-1, IL-6, IL-8, and TNF-a. C5a stimulates endothelial cells to release heparin sulfate, upregulate tissue factor, secrete von Willebrand factor and express P-selectin, which is conducive to fibrin deposition and which enhances thrombin-mediated platelet aggregation and polymorphonuclear leukocyte adhesion (23). Tissue factor and many adhesion molecules can be expressed by endothelial cells triggered by soluble C5b-9 (sC5b-9) (35). Complement activation may therefore induce direct inflammation and indirect effects mediated by endothelial cells.

In allogeneic transplantation, C3 is one of the important factors triggering rejection in mice (36, 37) and humans (38). Numerous studies have shown that the combination of C3 fragment C3dg with an antigen can be used as a strong adjuvant to promote both cellular and humoral responses (39). Therefore, it can be reasonably expected that complement activation and C3dg binding will trigger an immune response, leading to an adaptive immune response to the graft (34). Therefore, small interfering RNA (siRNA) targeting C3 and C5a receptors may increase the viability of transplanted islets (40).

HYPOXIC DAMAGE

Islets are easily damaged under hypoxic conditions prior to transplantation (including pancreas procurement, islet isolation and culture) and exposure to hypoxic environment of the transplant site after transplantation (41). Hypoxia damages islet β cell function, which manifested anaerobic glycolysis, showed elevated lactate and reduced responsiveness to high glucose levels (42). The decrease of blood oxygen partial pressure can lead to irreversible β cell dysfunction, resulting in higher fasting blood-glucose and lower C-peptide levels (43). Calcium influx into islet cells has also been shown to cause cell damage in rat and human islets cultured under hypoxic conditions (44). In order to cope with the impact of hypoxia on the quality and function of islets, the following explorations were attempted: (1) Compared with static cold storage, perfusion effectively reduced anoxic death of islet cells, and islet production was higher after perfusion (45, 46). Perfluorohexyloctan, a semi-fluorinated liquid fluorocarbon, maintained higher intrapancreatic pO2 and improved islet viability and function with porcine pancreas (47, 48). In addition, oxygen supply can be increased and oxygenation of islets can be improved by decreasing the culture density of islets before transplantation (46, 49). (2) Photosynthesis of thermostable microalga (Chlorella sorokiniana) was applied as a method to supply oxygen to cultured islets coencapsulating in alginate gel (50). Microparticle-mediated-oxygenation has been studied to improve islet transplantation.Co-transplantation of oxygen-generating microparticles and minimal islet mass within fibrin-conjugated heparin/VEGF collagen scaffold has enormous potential to enhance islet revascularization, diabetes reversal and oxygenation (51). (3) As mentioned above, the influx of calcium ions into islet cells can also induce islet injury. It was found that potassium channel activator (diazoxide) and calcium channel blocker (nifedipine) were helpful to restore the synthesis of insulin protoplasts and islet cell necrosis caused by hypoxia when used as preconditioning agents (44, 52). (4) Curcumin has the ability to protect β cells from hypoxia damage (53). Puerarin could alleviate β cell apoptosis and malfunction by hypoxic injury of β cells in corpulent mice induced by cobalt chloride induced *via* PI3K/Akt pathway activation (54). (5) Reconstructing the capillary network in islets is very important to prevent hypoxia and preserve function. Previous studies have shown the effectiveness of prevascularization of the graft bed for subcutaneous islet graft survival (55–58). The use of islet-cell cluster in clinical islet transplantation may be a strategy to prevent islet loss caused by hypoxia after transplantation (59).

IMMUNOSUPPRESSIVE DRUG TOXICITY AND IMMUNE TOLERANCE INDUCTION

During intraportal islet transplantation, isolated islets are exposed to high levels of immunosuppressive drugs, which are detrimental to islet engraftment/survival and long-term function (60). Previously, the standard protocol of immunosuppression

for islet transplantation included a combination of corticosteroids, calcineurin inhibitors (tacrolimus and cyclosporine), and purine analogs (mycophenolate mofetil). Many of the above drugs had been shown to be diabetogenic, impairing insulin secretion (61). The Edmonton protocol in 1999 took a major step forward in islet transplantation with the introduction of steroid-free therapy based on low-dose sirolimus, tacrolimus, and daclizumab (3). Although the results of multicenter clinical trials suggested that this protocol could provide short-term insulin independence and reduce the incidence of acute rejection (62), patients receiving long-term immunosuppressive drugs are susceptible to multiple adverse effects, such as infections (63), malignancies (64), *de novo* diabetes (65) and organ toxicities (66).

Immune tolerance induction is a promising strategy to accept histocompatibility complex (MHC)-mismatched allografts without reducing resistance to infection or increasing other complications (67). For immunological tolerance to allografts, the high proportion of MHC alloreactive T cell is considered as a major barrier to tolerance induction. Central T cell tolerance refers to the deletion of reactive clones in the thymus during negative selection. Peripheral T cell tolerance includes peripheral deletion, anergy/exhaustion, and suppressive function of regulatory T cells (Treg) (68). At present, how to apply the inherent immune tolerance mechanism of the human system to induce donor-specific immune tolerance is the key to solving transplant rejection (69). Singh et al. reported that apoptotic donor lymphocyte infusion prior to transplantation induced long-term tolerance (>1 year) of islet grafts in a non-human primate (NHP) model, which had made a breakthrough in the tolerance induction protocol for allogeneic islet transplantation (70).

Intrathymic inoculation of recipient APCs pulsed with allopeptides can induce intrathymic tolerance, however it is an invasive technique and the thymus regresses with age and has limited potential in adults (71). Another potentially more effective approach to achieving central tolerance is the generation of hematopoietic chimerism or mixed allogeneic chimerism, with lethal total body irradiation or sublethal total body irradiation (assisted by anti-CD4, CD8 monoclonal antibodies or costimulatory blockade) in the prospective transplant recipients, in order to make room for the transplanted bone marrow. Bone marrow cell transplantation can reconstitute the recipient's hematopoietic compartment with donor hematopoietic stem cells, inducing donor-specific tolerance to islet allografts (62, 72). Another strategy to induce tolerance is to deplete alloreactive T cells prior to transplantation, promoting a hyporesponsive environment that drives tolerance transition (73). T cell depletion can be achieved by total body irradiation, lymphocyte depleting alloantibodies. Among them, antithymocyte globulin (ATG) is a potent inducer of T cell depletion, and ATG alone or in combination with other drugs can prolong the survival of allografts (74, 75). Other pathways for inducing immune tolerance include costimulatory signal blockades, induction and expansion of regulatory T (Treg) cells, etc (62). And in the intrahepatic mouse allogeneic islet transplantation model, Lee et al. demonstrated for the first time that short-term single administration of anti-CD154 monoclonal antibody could induce FoxP3+ Treg cell-mediated immune tolerance (67).

KEY INFLAMMATORY FACTORS AND CELLS ASSOCIATED WITH ISLET CELL DYSFUNCTION

Several mediators have been found to cause islet dysfunction and/or cellular death after islet transplantation, including inflammatory cytokines (IL-1β, TNF-α, and IFN-γ), nitric oxide (NO), and nitric oxide synthase (iNOS) (76).

IL-1β

IL-1β is one of the most important mediators of islet injury and plays an important role in the process of pancreatic islets dysfunction, which may represent an early inflammatory marker of graft failure (77). IL-1β is secreted by Kupffer cells, islet resident macrophages, and neutrophils around the transplantation site (78). IL-1β secretion increases during islet acquisition, islet isolation, islet culture, and islet transplantation (77). IL-1β binds to the IL-1β receptor (IL-1βR) on the surface of pancreatic islet cells, causing TNF receptor-related factor 6 (TRAF6) to be activated by IL-1 receptor-related kinase (IRAK), which in turn leads to the phosphorylation and degradation of IκB. Then NF-κB is released from inhibitory IκB, transferred from the cytoplasm to the nucleus, and regulates the transcription of various genes, including IL-1, IL-6, TNF-α, and iNOS (76, 79) (**Figure 1**). Activation of iNOS results in the production of NO, which is directly related to β-cell apoptosis (80).

The damage of islet cells starts from the donor. Although most pancreatic islet transplants use organs from heart-beating brain-dead (BD) donors, acute physiological changes after brain death of BD donors may still cause significant damage to islets from inflammatory events. Brain death can stimulate various cells to produce pro-inflammatory cytokines, and produce a so-called "cytokine storm", including IL-1β in BD donors, which greatly reduces the islet yield, functionality, vitality, and engraftment after transplantation (8). One study has shown that the administration of exendin-4 to BD donors can reduce the expression of IL-1β, thereby increasing both the islet viability and insulin secretion in the pancreas after glucose stimulation in a BD rat model (81). In the BD rat model, treatment with a selective neutrophil elastase inhibitor, sivelestat sodium, decreased the expression of IL-1β, significantly improved the islet yield and function *in vitro*, and suppressed hypercytokinemia-mediated beta-cell death (82).

Then, in the process pancreas digestion and islet purification, enzymatic and mechanical stress can induce inflammatory mediators, such as IL-1β, in the islets (77). The islet basement membrane is lysed during the digestion and separation of the

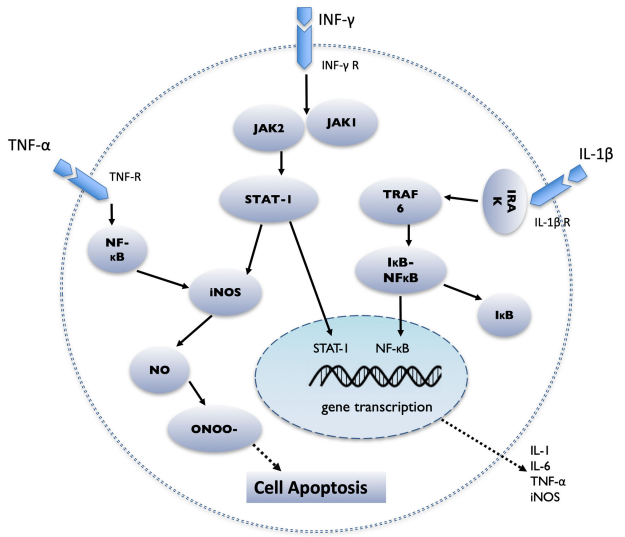

FIGURE 1 | Schematic diagram demonstrating the intracellular cascade of events that occur in the pancreatic islet after stimulation by IL-1β, IFN-γ and TNF-α. IL-1β binds to the IL-1β receptor (IL-1βR), causing TNF receptor-related factor 6 (TRAF6) to be activated by IL-1 receptor-related kinase (IRAK), then NF-κB is released from inhibitory IκB, transferred from the cytoplasm to the nucleus, and regulates the transcription of various genes, including IL-1, IL-6, TNF-α, and iNOS; TNF-α binding to the TNF receptor (TNF-R), initiates the activation of NF-κB and the induction of apoptosis; IFN-γ binds to the IFN-γ receptor (IFN-γR) and causes the activation of JAK1 and JAK2. JAK2 then activates signal transducers and activators of transcription 1 (STAT1), which then transfers to the nucleus, where it performs gene regulation.

pancreas, which interrupts the communication between islet basement membrane proteins and the integrins expressed by the islets (83). The loss of the interaction between the internal cells and the external microenvironment also interrupts the transmission of pro-survival signals (84). The isolation of pancreatic islets eventually leads to the increased expression of many stress kinases, which subsequently activates pro-inflammatory and pro-apoptotic pathways (43, 85). Furthermore, the functional clustering of differentially expressed genes revealed the upregulation of genes related to cell growth, angiogenesis, inflammation, and apoptosis after isolation and culture (43). A study has shown that islets in early culture (2 days) express more genes, including IL-1β, than islets in long-term culture (7-11 days). It seems that culturing islets before transplantation is beneficial for reducing the expression of inflammatory mediators (86). However, in cultured adult porcine islets, IL-1β mRNA was continuously detected at 1, 4, 8, and 11 days after isolation, and slightly increased over time (87). It is worth noting that a pretreatment culture with anakinra (IL-1 receptor antagonist) prior to human islet transplantation can improve the survival and function of human islets during culture (88).

The early damage of pancreatic islets is mainly manifested by an IBMIR, leading to a cytokine storm, involving IL-1β, TNF-α, and IFN-γ. These cytokine-activated macrophages produce IL-1β, which triggers the cytokine storm at the transplantation site, leading to a negative chain of events (89). Syngeneic transplant models have shown that the nonspecific inflammatory response increases IL-1β at the transplant site and affects early graft failure. In that study, the expression of IL-1β mRNA was maximal on day 1 after transplantation and then declined towards pre-transplantation levels on day 7 (90). Previous animal studies have indicated that approximately 60% of islet grafts lose their function *via* non-specific inflammation within 3 days after transplantation (91, 92). The overexpression of IL-1β receptor antagonist protein in transplanted islets can improve the outcome of the transplantation (93).

TNF-α

In humans, it appears that IL-1β must act in combination with IFN-γ and/or TNF-α (76). After TNF-α binds to the TNF receptor (TNF-R), it forms a trimer and undergoes conformational changes, which leads to the exposure of the intracellular death domain, and initiates the activation of NF-κB, the activation of the MAPK pathway, and the induction of apoptosis (40). Wen et al. revealed that TNF-α was significantly elevated in patients following allogeneic islet cell infusion compared with patients receiving autologous transplantation (94). Recently, multiple studies have shown that after pancreatic islet allotransplantation, the early use of a combination of anti-IL-1β (anakinra) and TNF-α (etanercept) inflammation blockade is beneficial for reducing islet damage caused by nonspecific inflammation and presumably led to better engraftment (95) (**Figure 1**).

IFN-γ

IFN-γ binds to the IFN-γ receptor (IFN-γR) and causes the activation of JAK1 and JAK2. JAK2 then activates signal transducers and activators of transcription 1 (STAT1), which then transfers to the nucleus, where it performs gene regulation (96). One study showed that IFN-γ transcripts were found in allografts at 1, 3, 5, and 7 days after transplantation, and peaked on day 5, but there were no such cytokines in syngeneic grafts (97). Some studies have shown that combined cytokines such as IL-1β, TNF-α, and IFN-γ lead to islet cell dysfunction or death (98).

NO AND INDUCIBLE NITRIC OXIDE SYNTHASE

Cytokines, such as IL-1β, TNF-α and IFN-γ, mainly stimulate the large expression of iNOS in β cells and macrophages to synthesize excessive NO, thereby causing damage to the pancreatic islets (99); the latter can form a highly active free radical peroxynitrite (ONOO-) by losing an electron and combining with superoxide free radicals, which have strong cytotoxicity and promote apoptosis (100, 101) (**Figure 1**). NO affects many physiological β-cell processes, including inhibition

of oxidative metabolism, changes in the expression of target genes, inhibition of glucose-stimulated insulin secretion, damage to DNA, and induction of endoplasmic reticulum (ER) stress. If exposure to NO is prolonged, it activates various signal cascades and eventually leads to the death of β cells (102). In xenogeneic islet transplantation animal models, selective iNOS inhibitors can suppress the production of induced NO, to prevent early islet graft failure (103).

IMMUNE CELLS

Macrophages

Macrophages, which are one of the cellular components of the innate immune system, show great heterogeneity in physiological and pathological conditions. They can be polarized into pro-inflammatory macrophages (M1) or anti-inflammatory macrophages (M2) in different environments (104). Lipopolysaccharide and interferon IFN-γ can activate M1 macrophages to secrete TNF-α, iNOS and superoxide anion to play pro-inflammatory and host defense functions. In contrast, M2 macrophages have a protective role in the immune response and inflammation. Other studies showed that M2 macrophages can be activated by interleukin (IL)-4 and IL-13, and play an immune regulatory and anti-inflammatory role, through the secretion of IL-1Rα, IL-1,TNF-α, IL-10, and other cytokines (104, 105).

After the islets are infused into the portal vein, the activated liver-resident macrophages (Kupffer cells) play a central role in the inflammatory responses within the liver, secreting a series of factors (including arachidonic acid metabolites, TNF-α, IFN-γ, IL-1, IL-6, complement, coagulation factors, reactive oxygen species and nitrogen) to recognize and respond to various signals from the surrounding microenvironment, directly affecting the survival of intrahepatic islets (11, 106, 107). Furthermore, Kupffer cells can induce a response of other non-immune cell subsets, including endothelial cells (108). In addition, it has been suggested that ischemia reperfusion phenomena may directly trigger the activation of sinusoidal endothelial cells and help trigger nonspecific inflammatory responses (109). The transplanted islets, residual endotoxin produced in the process of islet isolation, and liver sinusoidal endothelial damage can all activate Kupffer cells (11, 110). In addition, tissue damage can recruit inflammatory macrophages (M1), which in turn cause islet damage (111). At the same time, dying β cells also produce high mobility group box 1 (HGMB) and iNOS, attracting more macrophages to the liver, and enhancing inflammation and β cell death (14, 112) (**Figure 2**).

Macrophage depletion can significantly reduce the expression of IL-1β and TNF-α, indicating the role of macrophages in the production of inflammatory cytokines (11). Gou et al. demonstrated that Alpha-1 antitrypsin can protect the survival of islet grafts, in part by inhibiting the polarization of M1 macrophages both *in vivo* and *in vitro* (14). One study has mentioned that the local sustained-release of dexamethasone in grafts promotes the survival of mouse pancreatic islet grafts by inducing the differentiation of M2 macrophages in the graft microenvironment as well as the secretion of anti-inflammatory factors (104). Chappell et al. showed that activated M2 macrophages could improve the survival conditions of grafts in a mouse model by improving revascularization or neovascularization, which had a repair function in the graft reaction (113).

Lymphocytes

Type I diabetes is characterized by the autoimmune-mediated damage of islet β-cells, and the transplanted islets will also be

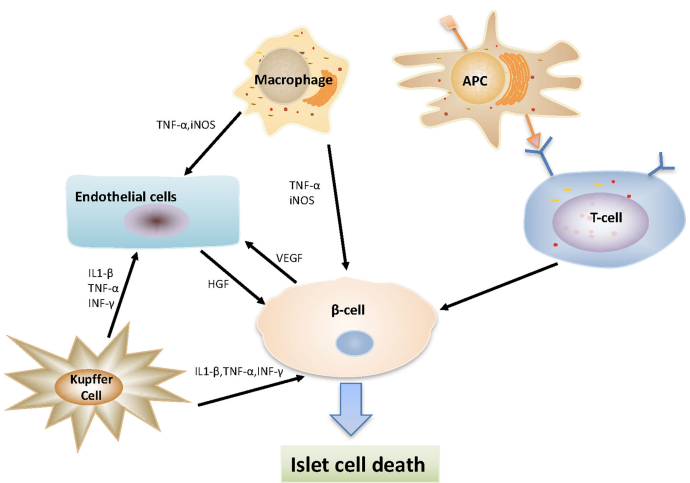

FIGURE 2 | Interaction between various types of cells after islet transplantation. Macrophages secreting TNF-α and iNOS, which act on beta-cells and endothelial cells to play a pro-inflammatory function; Kupffer cells secreting a series of substances (including IL-1β, IFN-γ and TNF-α), directly affecting the survival of intrahepatic islets and endothelial cells; Antigen presenting cells (APCs) take and process antigens from donor and present antigens to host T cells, then reactive CD8+, CD4+ T-cells destroy transplanted islet β cells.

attacked by the same stresses that destroy host β-cells (40). CD8+ T cells and CD4+ T cells are the major players in the destruction of β cells. Activated T cells produce cytokines such as IFN-γ, TNF-α, and lymphotoxin to induce β cell apoptosis. T cells also express ligands for the Fas receptor and TNF-related apoptosis-inducing ligands, both of which lead to apoptosis by activating effector caspases. In addition, CD8+ T cells directly contact and promote the release of granzyme B into the cytoplasm of target cells through perforin, thereby activating nucleases and caspases to kill the target cells (40, 98). In some studies, CD8+ T cell infiltration was observed in the pancreas of type 1 diabetic patients and the transplanted pancreas (114, 115). In mouse models, deficiency of CD4+ T cells was observed to stop progression to insulitis (116).

During the rejection of allogeneic transplantation, the host immune response can directly or indirectly be activated by T cells recognizing the donor tissue. Direct graft recognition involves an interaction between the donor tissue resident antigen-presenting cells (APCs) and host T cells *via* major histocompatibility complex (MHC) (117). Indirect recognition involves the treatment of donor graft peptides by host APCs and corresponding MHC interactions to stimulate host T cells (118). APCs, including macrophages, dendritic cells (DC), passenger leukocytes, from both donors and host are involved in the antigen presentation (40). The activation and maturation of T cells depends on the signals from the above APCs. If these signals are blocked, T cells will undergo apoptosis (**Figure 2**).

DCs can not only initiate an immune response, but also induce central or peripheral immune tolerance (40). Mature or activated DCs can initiate a positive immune response, while immature DCs or DC precursors show tolerance. NF-ĸB is a transcription factor that is necessary for DC differentiation and maturation. The inhibition of the NF-ĸB pathway has been shown to produce tolerogenic DCs (119). RelB is a major NF-ĸB protein, which inhibits the expression of MHC-II, CD80, and CD86, and ultimately prevents the maturation of dendritic cells. RelB silenced DCs can inhibit antigen-specific alloreactive immune rejection and reduce the proliferation of antigen-specific T cells (120). In another study, tolerogenic DCs were generated by inhibiting the expression of CD80 or CD86. The administration of modified dendritic cells (DC) can prolong the survival of allografts, thereby inducing T cell hyporesponsiveness and apoptosis (121).

Granulocytes

The poor outcome of intraportal islet transplantation can be explained by IBMIR, one of which is characterized by leucocyte infiltration. The islets were mixed with ABO-compatible blood in a heparinized tube, and the first neutrophilic granulocytes appeared in the islets after 15 minutes, increasing at 1 hour and peaking at 2 hours (122). Neutrophilic granulocytes induce cell damage through cytotoxic attack and phagocytosis. After neutrophil activation, superoxidase is produced to form reactive oxygen species (ROS) and release protease, both of which are involved in killing microbes (123). Neutrophilic granulocytes are also known to contain a large number of cytokines, which are released upon activation, and there is much evidence that cytokines have a damaging effect on pancreatic islets (124). And their infiltration results in the release of chemokines such as TNF-α and macrophage inflammatory protein 1α (MIP-1α) from T cells and macrophages. The mobilization of this immune effector may have effects on specific immune systems, inducing and enhancing cellular rejection (122). The massive infiltration of neutrophilic granulocytes not only causes functional impairment or reduces the mass of the implanted islets, but may also amplify the subsequent immune response, causing direct damage to the islets (125). Therefore, the development of drugs targeting neutrophil toxicity may markedly improve the outcome of intraportal islet transplantation.

Endothelial Cells

Pancreatic islets have an extensive capillary network, which—in addition to providing nutrients and oxygen to the islet endocrine cells and transporting hormones to the peripheral circulation—is an important source of signals for improving the survival rate and function of islet β cells (126). While islets only constitute 1% of the weight of the pancreas, they receive approximately 15% of the blood flow of the pancreas (127). In the process of the isolation, the islets are disrupted from the surrounding tissue and the capillary networks. Therefore, revascularization of the islets after transplantation is important for the function and survival of the islet graft. The endothelial cells retained in the islets after islet isolation are lost after the islets are cultured for 7 days. The isolated islets are considered to be an avascular tissue, and revascularization is carried out through the blood vessels that grow from the host organ to the islets (128). Angiogenesis begins on the first day after transplantation, and vascular remodeling may continue for up to 3 months (129). In comparison to cultured islets, transplantation of freshly isolated islets containing numerous endothelial cells may significantly improve the vascularization of transplanted islets, which in turn leads to an enhanced endocrine function and the survival of islet grafts (130). Olsson et al. observed that islet grafts obtained from freshly isolated islets have higher vascular density and oxygen tension, as well as higher ability to cure chemically induced diabetes, in comparison to islet grafts obtained from cultured islets (131).

Pancreatic islets and vascular endothelial cells secrete high levels of vascular endothelial growth factor (VEGF), which can recruit neovascularization (132). Cheng et al. used adenovirus containing cDNA from human VEGF isoforms to transfected islets and transplanted it into diabetic nude mice. It was found that the blood glucose was normal and that the revascularization of islets was improved (133). Johansson et al. demonstrated that the production of hepatocyte growth factor (HGF) *in vitro* by endothelial cells increased the proliferation of beta cells, which in turn required VEGF from beta cells (134). VEGF can also stimulate the release of interleukins and increase blood flow to ischemic tissues (135) (**Figure 2**). However, the supplementation of VEGF in islet grafts may have a negative impact, which recruits and amplifies inflammation, which may destroy islets (133). Previous *in vitro* and *in vivo* studies have shown that resident liver macrophages and endothelial cells can mediate

early islet dysfunction by secreting cytokines and activating inducible iNOS (103).

CONCLUSION

Islet transplantation remains a promising treatment to improve the quality of life for many individuals with type 1 diabetes. If the restoration of normal glucose tolerance can be achieved, non-specific inflammation during transplantation can be reduced, and immune tolerance to islet tissue can be induced, it will be an ideal treatment for this disease. However, during the process of islet transplantation—including isolation, culture, and islet implantation—inflammation, ischemia, hypoxia, and immune responses will occur, resulting in the loss of the graft. The transplantation microenvironment plays an important role. Cytokine-mediated non-specific inflammation, immune cell-mediated rejection, and endothelial cells participate in post-transplant vascular remodeling, which directly and indirectly affect graft survival. Some cytokine inhibitors and siRNA targeting complement receptors have been shown to improve the viability of transplanted islets. Understanding the influence of the microenvironment on the survival of transplanted islets, as well as possible therapeutic targets is significant for the future of islet transplantation. The improvement of the microenvironment and the continuous progress of transplantation strategies will eventually improve the prognosis of transplant recipients.

AUTHOR CONTRIBUTIONS

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Both Natural and Induced Anti-Sda Antibodies Play Important Roles in GTKO Pig-to-Rhesus Monkey Xenotransplantation

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Sda, produced by the B4GALNT2 enzyme, has been recognized as an important xenoantigen for pig-to-nonhuman primate xenotransplantation. However, little is known about Sda expression in pigs and its immunogenicity in xenotransplantation. In this study, peripheral blood mononuclear cells (PBMCs) were isolated from wildtype, GTKO (with high, moderate, and low Sda expression), GTKO/β4GalNT2KO, GTKO/CMAHKO, or GTKO/CMAHKO/β4GalNT2KO pigs. Anti-pig IgM/IgG binding and complement-dependent cytotoxicity (CDC) to pig PBMCs was measured by flow cytometry using pooled rhesus monkey sera (n=20) or human sera (n=20). As compared to wild-type pigs (n=12), GTKO pigs (n=17) had a significantly higher mean level of Sda expression on PBMCs and showed a greater individual difference in expression. Both the overall binding of monkey serum IgM/IgG antibody to GTKO pig PBMCs and CDC against these PBMCs decreased significantly with a progressive reduction in Sda expression, showing a clear dose-effect relationship. Both the monkey serum antibody binding and CDC decreased significantly after the additional deletion of Sda, whereas the binding of human serum antibody and CDC against the GTKO pig PBMCs were markedly reduced after the deletion of Neu5Gc in the pigs. In addition, anti-Sda antibody accounted for > 50% of the induced anti-non-Gal antibody at the time of rejection in two rhesus monkeys that received GTKO/hCD55 pig kidney xenotransplantation, and the anti-Sda antibody showed significant cytotoxic activity against GTKO pig cells. We conclude that both natural and induced anti-Sda antibodies play important roles in GTKO pig-to-rhesus monkey xenotransplantation, thus providing further evidence for GTKO/β4GalNT2KO pigs as the preferred organ source for rhesus monkeys as a preclinical model of xenotransplantation.

Keywords: Sda, xenotransplantation, genetic engineering, pig, rhesus monkey

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INTRODUCTION

Organ shortage remains a major problem for clinical organ transplantation worldwide (1). Xenotransplantation using genetically engineered pigs as organ donors is considered a promising way to solve this problem (2–4). To date, three carbohydrate xenoantigens expressed on porcine cells have been shown to play important roles in pig-to-human xenotransplantation: galactose-α1,3-galactose (Gal), N-glycolylneuraminic acid (Neu5Gc), and Sda. These xenoantigens can be deleted individually or in combination by using gene editing techniques (5–17). At present, a variety of genetically modified pigs have emerged, among which triple-knockout (TKO) pigs (GTKO/β4GalNT2KO/CMAHKO) are considered to be the best choice for future clinical trials (2–4, 11, 15, 16).

Preclinical studies using Old World nonhuman primates (NHP) as xenograft recipients had been standard practice for many years before pig-to-human xenotransplantation could become a reality. Baboons, rhesus monkeys, and cynomolgus monkeys have been the most commonly used Old World NHPs for preclinical xenotransplantation studies. Unlike humans, these Old World NHPs express Neu5Gc as pigs do, so they do not generate natural antibodies against Neu5Gc. Because of this difference, TKO pigs are not necessarily ideal donors for preclinical studies in the Old World NHPs. Several *in vitro* and *in vivo* studies have indicated that xenotransplantation into Old World monkeys or baboons using TKO pig donors is likely to be less effective than using GTKO/β4GalNT2KO pig donors, and even worse than using GTKO pig donors (11, 15–17). Andrew B. Adams' group has reported that pre-transplant selection of recipients with low titers of anti-pig antibodies significantly prolongs renal xenograft survival in a GTKO/β4GalNT2KO pig-to-rhesus monkey kidney transplant model (12, 18). Therefore, GTKO/β4GalNT2KO pigs have been suggested as the preferred organ source for Old World NHPs as a preclinical model of xenotransplantation (17).

In recent years, the use of GTKO/β4GalNT2KO pigs has confirmed the presence of preformed anti-Sda antibodies in Old World NHPs and humans (12). However, although the Sda antigen has been known for many years, little is known about the expression and immunogenicity of the glycan produced by the porcine β4GalNT2 enzyme. In order to further clarify the important role of Sda in pig-to-rhesus monkey xenotransplantation, we have now designed *in vitro* experiments to investigate (i) the differences in Sda expression on peripheral blood mononuclear cells (PBMCs) in individual wildtype (WT) pigs and GTKO pigs as well as the two populations as a whole; (ii) the impact of different levels of Sda expression on monkey serum IgM/IgG antibody binding and complement-dependent cytotoxicity (CDC) against pig PBMCs with Gal deletion; (iii) potential differences in the role of Sda in pig-to-rhesus monkey vs. pig-to-human xenotransplantation; and (iv) whether the expression of Sda can induce a significant antibody response after GTKO pig-to-rhesus monkey xenotransplantation and thus play an important role in the development of acute humoral xenograft rejection.

Abbreviations: APC, allophycocyanin; CDC, complement-dependent cytotoxicity; CMAH, CMP-N-acetylneuraminic acid hydroxylase; DBA, Dolichos biflorus agglutinin; GTKO, α1,3-galactosyltransferase gene knockout; NHP, nonhuman primate; PBMCs, peripheral blood mononuclear cells; TKO, triple-knockout; β4GalNT2, β1,4-N-acetyl-galactosaminyl transferase 2.

MATERIALS AND METHODS

Sources of Rhesus Monkey and Human Sera

Monkey sera were obtained from 20 outbred male rhesus monkeys (4-10 years old, Hubei Tianqin Biotechnology Corporation and South China Primates Research Center, Hubei, China). Human sera were obtained from 20 healthy human volunteers (22–44 years old, both genders) who had no history suggesting previous exposure to pig antigens or to alloantigens (such as blood transfusions, a previous failed renal allograft, or pregnancy). Monkey or human sera were stored in as either single or pooled samples at −80°C. When required, decomplementation was carried out by heat-inactivation for 30 min at 56°C.

We had previously performed GTKO pig-to-rhesus monkey kidney xenotransplants in another study; two of the recipients had developed acute humoral xenograft rejection and graft failure at 19 days post-transplant. The recipients' sera collected 6 days before transplantation and at the time of rejection (day 19) were stored at -80°C and were used in the present study.

Sources of Pig Cells

PBMCs were obtained from WT (n=12); α1,3-galactosyltransferase gene knockout (GTKO) (n=17); α1,3-galactosyltransferase/β1,4-N-acetyl-galactosaminyl transferase 2 gene double-knockout (GTKO/β4GalNT2KO) (n=1); α1,3-galactosyltransferase/CMP-N-acetylneuraminic acid hydroxylase gene double-knockout (GTKO/CMAHKO); (n=1) or α1,3-galactosyltransferase/β1,4-N-acetyl-galactosaminyl transferase 2/CMP-N-acetylneuraminic acid hydroxylase gene triple-knockout and human CD55 transgenic (GTKO/β4GalNT2KO/CMAHKO/hCD55; TKO/hCD55, n=1) pigs (Chengdu Clonorgan Biotechnology Co., LTD, Chengdu, China), all of blood type O and the same genetic background (Bama miniature pig). The pig PBMCs from the various donors were isolated by Ficoll density gradient centrifugation as described (19). Representative phenotypic identification results are shown in **Figure 1**. To study the role of Sda expression in pig-to-rhesus monkey xenotransplantation, two wild-type pigs with a high (WT-Sda^{hi}) or low (WT-Sda^{lo}) level of Sda expression and three GTKO pigs with high (GTKO-Sda^{hi}), moderate (GTKO-Sda^{mo}) or low (GTKO-Sda^{lo}) Sda expression were selected to provide PBMCs for related *in vitro* experiments. Although the expression of human complement regulatory protein in transgenic pigs would affect the complement-dependent cytotoxicity (CDC) results, because we were unable to obtain a pure GTKO/β4GalNT2KO/CMAHKO (TKO) pig, we used a TKO/hCD55 pig instead.

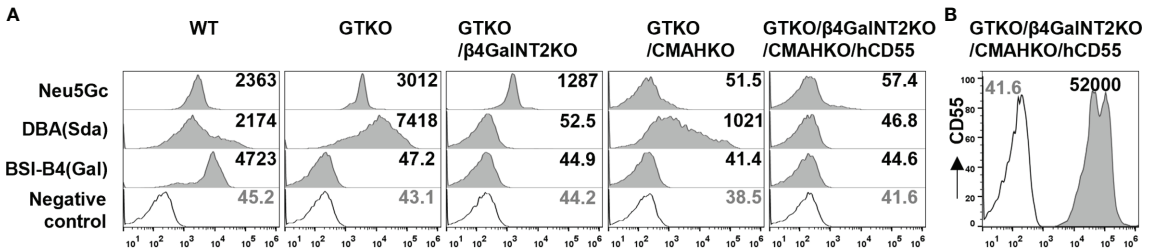

FIGURE 1 | Expression of Gal, Sda, and Neu5Gc on pig PBMCs. **(A)** The expression of Gal, Sda, and Neu5Gc on pig PBMCs from wildtype (WT), GTKO, GTKO/β4GalNT2KO, GTKO/CMAHKO, and TKO/hCD55 pigs (Gmean values were shown). White histograms outlined in black represent negative controls that were unstained cells for lectin experiments. **(B)** The expression of human CD55 (hCD55) on TKO/hCD55 pig PBMCs.

Detection of the Expression of Gal, Sda, Neu5Gc, and Human CD55 on Porcine PBMCs by Flow Cytometry

PBMCs were isolated from the peripheral blood of each pig, and 100 μl of cell suspension containing 5×10^5 PBMCs were dispensed into each FACS tube. Pig PBMCs were stained for the expression of Gal (by isolectin BSI-B4, 1:500, Sigma-Aldrich, St. Louis, MO), Sda (with Dolichos biflorus agglutinin, DBA, 1:1000, Vector Labs, Burlingame, CA, USA), and Neu5Gc (with chicken anti-Neu5Gc mAb, 1:800, Biolegend, San Diego, CA). The expression of human CD55 on PBMCs from the TKO/hCD55 pig was detected by using allophycocyanin (APC)-conjugated anti-human CD55 (1:100, Biolegend, San Diego, CA). Samples were analyzed on a flow cytometer (Cytoflex S, Beckman Coulter, USA), and the data were analyzed using FlowJo software (Treestar, San Carlos, CA). The results are reported as geometric mean fluorescence (Gmean).

Detection of the Binding of Serum IgM and IgG to Pig PBMCs by Flow Cytometry

Serum IgM and IgG binding to pig PBMCs were measured by flow cytometry, using a technique similar to that previously described (19, 20). In brief, 100 μl of isolated pig PBMCs (5×10^5 cells/tube) were incubated with 100 μl diluted rhesus monkey or human serum (10% final concentration, heat-inactivated for 30 min at 56°C in advance) for 30 min at 4°C. Cells incubated with 100 μl of phosphate-buffered saline (PBS) served as negative controls. After incubation, the cells were washed twice in PBS and centrifuged at 400 g for 5 min. The supernatant was discarded. To prevent non-specific binding, 200 μl of 10% heat-inactivated goat serum (Solarbio, Beijing, China) was added, and incubation was performed for 15 min in the dark at 4°C. After a wash with FACS buffer, the cells were incubated for 30 min with donkey anti-human IgM (1:1600) or goat anti-human IgG (1:1600) conjugated to Alexa-488 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) or APC-conjugated mouse anti-human IgG (1:1000, Biolegend, San Diego, CA). After incubation, the samples were washed twice and then resuspended with 200 μl PBS. The stained cells were analyzed by flow cytometry (Cytoflex S, Beckman Coulter, USA), and the data were analyzed using FlowJo software (Treestar, San Carlos, CA). The results were expressed as the Gmean. Each sample was repeatedly measured in three independent experiments.

Complement-Dependent Cytotoxicity (CDC) Assay for PBMCs

CDC assays were performed and monitored by flow cytometry using WT-Sda^{hi}, WT-Sda^{lo}, GTKO-Sda^{hi}, GTKO-Sda^{mo}, GTKO-Sda^{lo}, GTKO/β4GalNT2KO, GTKO/CMAHKO, or TKO/hCD55 pig PBMCs as target cells, as described in detail (19). In brief, pig PBMCs (5×10^5 cells in 100 μl FACS buffer) were incubated with 100 μl of diluted serum for 1 h at 4°C. The final serum concentration varied from 50% to 1.57%. After a wash with FACS buffer, 50 μl of rabbit complement (Cedarlane, Hornby, CA, USA) were added to each tube and incubated for 30 min at 37°C. After the incubation, the cells were washed with FACS buffer, and then 100 μl of propidium iodide (2 μg/ml, eBioscience, Inc., CA, USA) were added to each tube. After further incubation in the dark for 15 min at 4°C, the cells were examined by flow cytometry (Cytoflex S, Beckman Coulter, USA). The results were analyzed using FlowJo software, and the percentage of propidium iodide-positive cells was used to determine the extent of the CDC that occurred.

Statistical Analysis

Data are presented as means ± SD. The variation of individuals within each group was indicated by coefficient of variation (C.V=SD/mean x 100%). The significance of the difference between two groups was determined by unpaired *t*-test. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. All statistical analyses were performed using Graph Pad Prism version 7 software (GraphPad Software, USA). $P<0.05$ was considered statistically significant.

RESULTS

Expression of Gal, Sda, and Neu5Gc on PBMCs From Different Types of Knockout Pigs

We first assessed the expression of three carbohydrate xenoantigens (Gal, Sda, and Neu5Gc) in pigs with different types of gene

knockout. FACS analysis revealed that 1) PBMCs from wild-type pigs expressed all three xenoantigens; 2) GTKO pig PBMCs expressed Sda and Neu5Gc, but not Gal; 3) PBMCs from GTKO/β4GalNT2KO pigs did not express Gal or Sda; 4) PBMCs from GTKO/CMAHKO pigs did not express Gal or Neu5Gc; and 5) TKO/hCD55 pig PBMCs did not express any of the three xenoantigens (**Figure 1**).

The Expression of Sda in WT or GTKO Pig PBMCs

To determine whether there was a difference in Sda expression between wild-type and GTKO pigs and whether there were large individual differences within each population, we performed FACS assays to detect Sda expression on PBMCs from 12 wild-type pigs and 17 GTKO pigs. As shown in **Figure 2A**, there was a relatively low individual variation in the expression of Sda in wild-type pigs (Gmean: 1094-3345, SD=690, C.V=32.4%), but the individual variation in Sda expression in GTKO pigs was much greater (Gmean: 140-8465, SD=2175, C.V=60.1%). As compared to wild-type pigs, GTKO pigs had a higher mean level of Sda expression on their PBMCs (Gmean: 3618 ± 2175 vs., 2130 ± 690, p=0.03).

To study the role of Sda expression in pig-to-rhesus monkey xenotransplantation, we selected two wild-type pigs and three GTKO pigs with different levels of Sda expression to provide PBMCs for our subsequent *in vitro* experiments. The expression of Sda on the PBMCs from the two wild-type pigs was high (WT-Sda^{hi}) in one case and low (WT-Sda^{lo}) in the other, and the expression of Sda on the PBMCs of the three GTKO pigs was high (GTKO-Sda^{hi}) in one case, moderate (GTKO-Sda^{mo}) in the second, and low (GTKO-Sda^{lo}) in the third (**Figure 2B**).

Natural Anti-Pig Antibody Levels in Serum of Rhesus Monkeys Show Great Individual Differences

To determine whether there are large individual differences in the levels of preformed anti-Gal and anti-Sda antibodies in rhesus monkeys, we randomly selected 5 of 20 rhesus monkeys that had not been immunologically screened, and measured both anti-pig IgM and IgG levels in the serum of each monkey by flow cytometry using PMBCs from WT (Sda^{hi} and Sda^{lo}), GTKO (Sda^{hi}, Sda^{mo}, and Sda^{lo}) or GTKO/β4GalNT2KO pigs as the source of the target cells. When PBMCs from WT pigs were used as the target cells for antibody detection, the levels of both anti-pig IgM and IgG in serum of the five monkeys showed great individual differences, regardless of the high or low expression of Sda on the porcine PBMCs (SD values of the WT-Sda^{hi} and WT-Sda^{lo} groups were 1620 and 1550 (IgM), 537.3 and 538.6 (IgG), respectively), suggesting that there were large individual differences in the levels of anti-Gal antibodies (**Figure 3**). When PBMCs from GTKO pigs were used as the target cells for antibody detection, the results reflected the level of antibodies against non-Gal antigens. As shown in **Figure 3**, the anti-non-Gal IgM levels showed an increasingly smaller individual difference as the Sda expression decreased on the porcine PBMCs, whereas the individual differences in the IgG levels did not show a similar pattern (from left to right, SD values of IgM were 892.7, 447.1, 194.7, and 122.8; SD values of IgG were 234.9, 223.6, 261.9, and 200.4). These results suggest that, at the least, the levels of serum anti-Sda IgM antibodies vary greatly among different rhesus monkeys. In order to avoid interference caused by these individual differences, we mixed serum samples from 20 monkeys together for subsequent studies.

Sda Is the Major Xenoantigen for the Binding of Monkey Serum Antibody to GTKO Pig PBMCs and Plays an Important Role in CDC Against These Cells

The IgM/IgG antibody binding and CDC were measured by flow cytometry after the co-culture of the pooled monkey sera with PMBCs from wildtype (Sda^{hi} and Sda^{lo}), GTKO (Sda^{hi}, Sda^{mo}, and Sda^{lo}), or GTKO/β4GalNT2KO pigs. As shown in **Figure 4A**, both the antibody binding (IgM and IgG) and CDC against WT pig PBMCs were very high (GMean of IgM: ~5000; GMean of IgG: ~4000; CDC: >90%), regardless of the high or low expression of Sda on the porcine PBMCs. In contrast, the binding of serum IgM

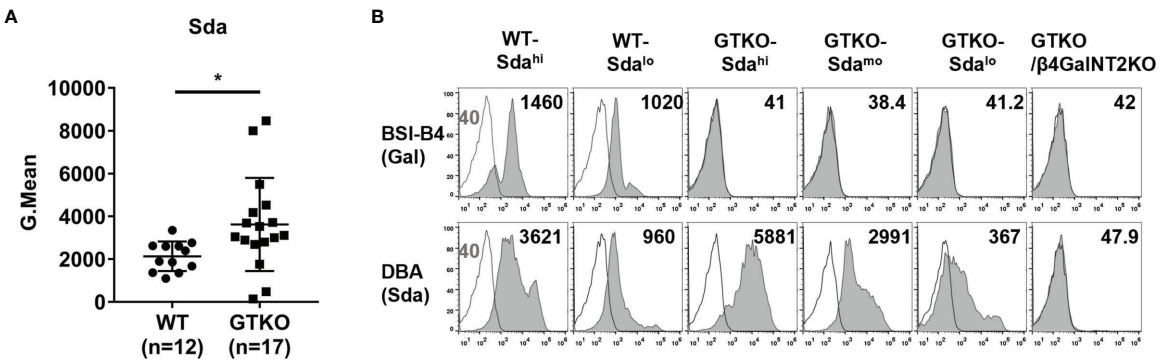

FIGURE 2 | The expression of Sda in WT or GTKO pigs. PBMCs from WT, GTKO, or GTKO/β4GalNT2KO pigs were stained for the expression of Gal (by isolectin BSI-B4) and Sda (with Dolichos biflorus agglutinin, DBA). The results are reported as geometric mean fluorescence (Gmean). **(A)** Comparison of the expression of Sda on WT (n=12) and GTKO (n=17) pig PBMCs. Data are presented as means ± SD (*P<0.05); **(B)** The expression of Gal and Sda on PBMCs from two wild-type pigs and three GTKO pigs with different levels of Sda expression. White histograms outlined in black represent negative controls that were unstained cells for lectin experiments. The appearance of a single histogram in GTKO or GTKO/β4GalNT2KO samples indicates an overlap with the negative control.

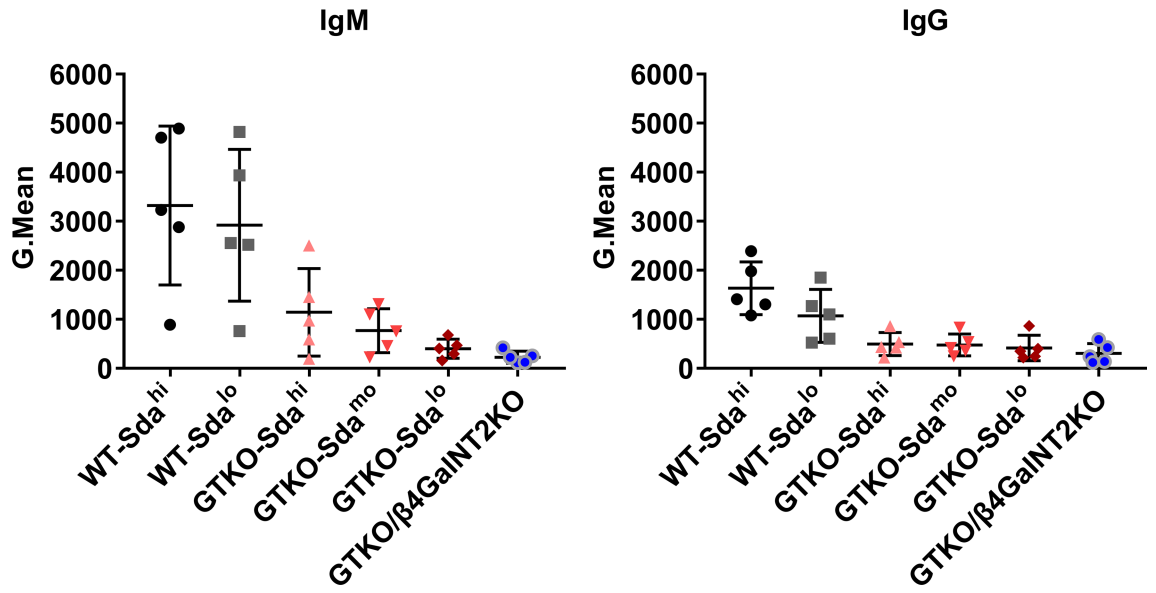

FIGURE 3 | Natural anti-pig antibody levels in the serum of rhesus monkeys. Five of 20 rhesus monkeys that had not been immunologically screened were randomly selected. Both anti-pig IgM and IgG levels in the serum (diluted at 1:10) of each monkey were measured by flow cytometry using PMBCs from WT (Sda^{hi} and Sda^{lo}), GTKO (Sda^{hi}, Sda^{mo}, and Sda^{lo}) or GTKO/β4GalNT2KO pigs as the source of the target cells. The results are reported as Gmean. Data are presented as means ± SD.

and IgG antibody to PBMCs from GTKO pigs was significantly lower than that to WT pig PBMCs ($P<0.01$). These results confirm that Gal is still the most important xenoantigen in pig-to-monkey xenotransplantation.

Notably, the monkey serum IgM/IgG binding to GTKO pig PBMCs (Gal-negative) with high expression of Sda remained relatively high (GMean of IgM: ~2700; GMean of IgG: ~1000), a level of reactivity that could still mediate a high level of CDC (>90%) at a serum concentration from 12.5% to 50%. When compared to the results obtained using GTKO pig PBMCs with different levels of Sda expression (high, moderate, low, negative) as target cells, the overall monkey serum IgM/IgG antibody binding and CDC to pig PBMCs decreased significantly with the reduction in Sda expression (**Figure 4B**). This result was confirmed by using PBMCs from another group of GTKO pigs with high, medium and low Sda expression (**Supplemental Figure 1**). The level of CDC against GTKO pig PBMCs with low expression of Sda was almost the same as that to GTKO/β4GalNT2KO pig PBMCs ($P>0.05$) (**Figures 4B, C**). These *in vitro* results suggest that the monkey serum-mediated CDC against GTKO pig PBMCs is closely related to the expression level of Sda; thus, Sda may play a major role in GTKO pig-to-rhesus monkey xenotransplantation.

In the Absence of the Gal Antigen, Human Antibody Binding to Porcine PBMCs Is Dependent on the Presence of Neu5Gc, Whereas Monkey Antibody Binding Depends on Sda

Next, we investigated the importance of various porcine xenoantigens in transplantation into humans and rhesus monkeys. For this purpose, IgM/IgG antibody binding and CDC were measured by flow cytometry after co-culture of pooled human or monkey sera with PMBCs from WT, GTKO, GTKO/β4GalNT2KO, GTKO/CMAHKO, or TKO/hCD55 pigs. In the case of both humans and rhesus monkeys, the binding of serum IgM and IgG to GTKO pig PBMCs (Gal deletion) was significantly lower than that to wild-type pig PBMCs ($P<0.01$) (**Figures 5A, B, D, E**). The binding of human serum IgM to GTKO/β4GalNT2KO pig PBMCs (both Gal and Sda deletion) was slightly lower than that to GTKO pig PBMCs ($P<0.05$), but there was no significant change in serum IgG binding ($P>0.05$). In contrast, both human serum IgM and IgG binding to GTKO/CMAHKO pig PBMCs (both Gal and Neu5Gc deletion) was dramatically lower than that to GTKO pig PBMCs ($P<0.01$) and almost close to the binding levels to TKO/hCD55 pig PBMCs (Gal, Sda, and Neu5Gc deletion) (**Figures 5A, B**). In addition, the cytotoxicity of human serum against porcine PBMCs decreased only at lower serum concentrations (6.25%-1.57%) after the deletion of Gal alone or both Gal and Sda in pigs, whereas the cytotoxicity of human serum against porcine PBMCs was markedly decreased at all serum dilutions after the deletion of Gal and Neu5Gc in pigs (**Figure 5C**). These results suggest that next to anti-Gal antibody, anti-Neu5Gc antibody is the main preformed anti-pig antibody in human serum.

Unlike the results we obtained for pooled human serum, the binding of rhesus monkey serum IgM antibody to GTKO/β4GalNT2KO pig PBMCs was significantly lower than that to pig PBMCs from WT, GTKO, GTKO/CMAHKO, or even TKO/hCD55 pigs ($P< 0.01$ or 0.001) (**Figure 5D**). The binding of monkey serum IgG antibody to GTKO/β4GalNT2KO pig PBMCs was also significantly lower than that to pig PBMCs from WT,

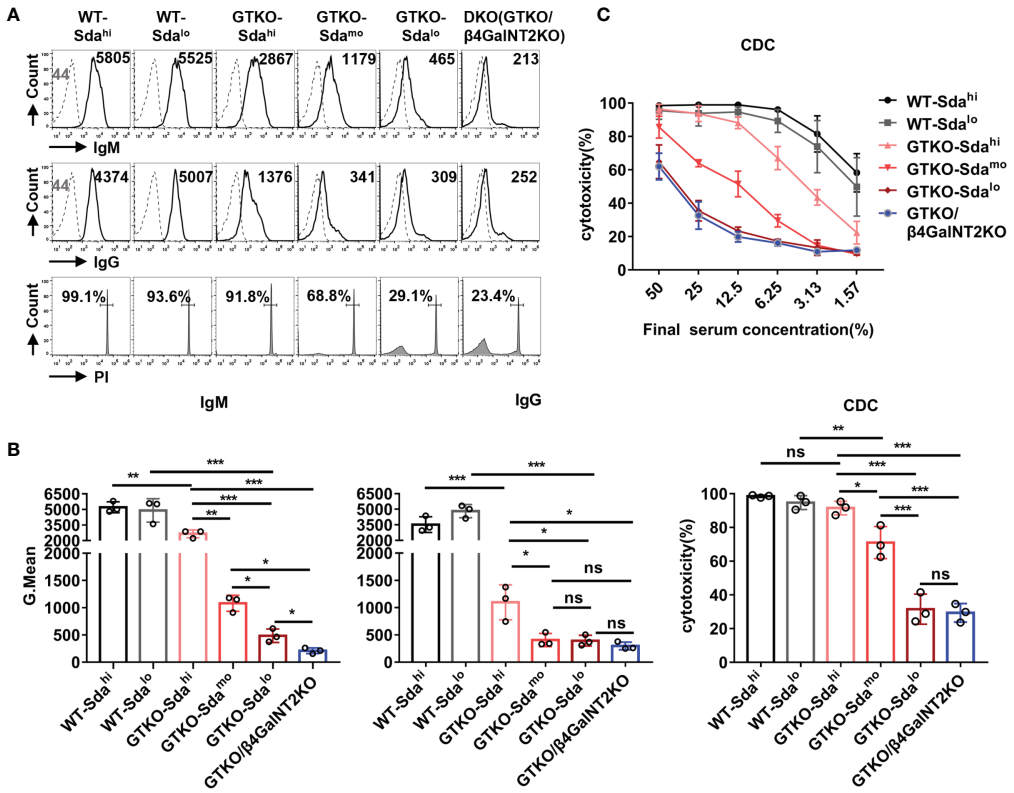

FIGURE 4 | The role of Sda expression in the binding of monkey serum antibody to GTKO pig PBMCs and in CDC against these cells. The IgM/IgG antibody binding and CDC were measured by flow cytometry after the co-culture of the pooled monkey sera with PMBCs from wildtype (Sda^{hi} and Sda^{lo}), GTKO (Sda^{hi}, Sda^{mo}, and Sda^{lo}), or GTKO/β4GalNT2KO pigs. **(A)** Flow cytometric histograms showing representative binding of rhesus monkey IgM/IgG antibody (top/middle row, serum diluted 1:10, Gmean values were shown) to pig PBMCs and CDC (bottom row, serum diluted 1:6, percentages were shown) against the same PBMCs. **(B)** Statistical analysis of pooled rhesus monkey serum antibody binding (IgM and IgG) to pig PBMCs and CDC against the same PBMCs. **(C)** Comparison of mean serum CDC (at serum concentrations of 50% to 1.57%) of pooled rhesus monkey against WT, GTKO, or GTKO/β4GalNT2KO pig PBMCs. Each sample was measured three times. All data are presented as means ± SD (*P<0.05, **P< 0.01, ***P< 0.001; ns, non-significant). The P value comes from three independent experiments.

GTKO, or GTKO/CMAHKO pigs (P<0.05 or 0.01) but similar to the binding to TKO/hCD55 pig cells (**Figure 5E**). Consistent with the antibody binding results, the cytotoxicity of monkey serum against GTKO/β4GalNT2KO pig PBMCs was significantly lower than that against WT, GTKO, GTKO/CMAHKO, or even TKO/hCD55 pig PBMCs (**Figure 5F**). These results indicate that Sda is more important for pig-to-rhesus monkey xenotransplantation, and GTKO/β4GalNT2KO pigs are a more suitable organ source than the other pigs for rhesus monkeys as a preclinical model of xenotransplantation.

Induced Antibodies Against Sda Play an Important Role in the Development of Acute Humoral Xenograft Rejection in GTKO/hCD55 Pig-to-Rhesus Monkey Kidney Xenotransplantation

We have performed four renal xenotransplants in rhesus monkeys using kidneys from GTKO/hCD55 pigs. The recipient monkeys received induction therapy with anti-thymocyte globulin (thymoglobulin) and anti-CD20mAb (rituximab) and maintenance therapy with tacrolimus, mycophenolate mofetil, and low-dose corticosteroids. Two of the four recipient monkeys (R01 and R02) generated high levels of circulating antibodies against non-Gal antigens and developed acute humoral xenograft rejection on day 19 (data not shown). In order to study the role of anti-Sda antibody in xenograft rejection, we used serum samples collected before transplantation (day -6) and at the time of rejection (day 19) to measure IgM/IgG antibody binding and CDC against GTKO or GTKO/β4GalNT2KO pig PBMCs.

As shown in **Figure 6**, the binding of IgM and IgG antibodies to GTKO pig PBMCs was significantly (4 to 14 times) higher in the serum of both monkeys on post-transplant day 19 than in serum collected before transplantation (day -6), indicating that a large proportion of the antibody against non-Gal antigens was newly generated after transplantation. To analyze whether these anti-non-Gal antibodies contain a significant amount of anti-Sda antibodies, we compared the results of antibody binding assays using GTKO pig PBMCs (Sda^{mo}) and GTKO/β4GalNT2KO pig PBMCs as target cells. In the sera of both monkeys at 19 days after transplantation, the levels of IgM and IgG binding to GTKO/β4GalNT2KO pig PBMCs were significantly lower than those to GTKO pig PBMCs (IgM decreased by 53% and 54%, IgG decreased by 81% and 50%,

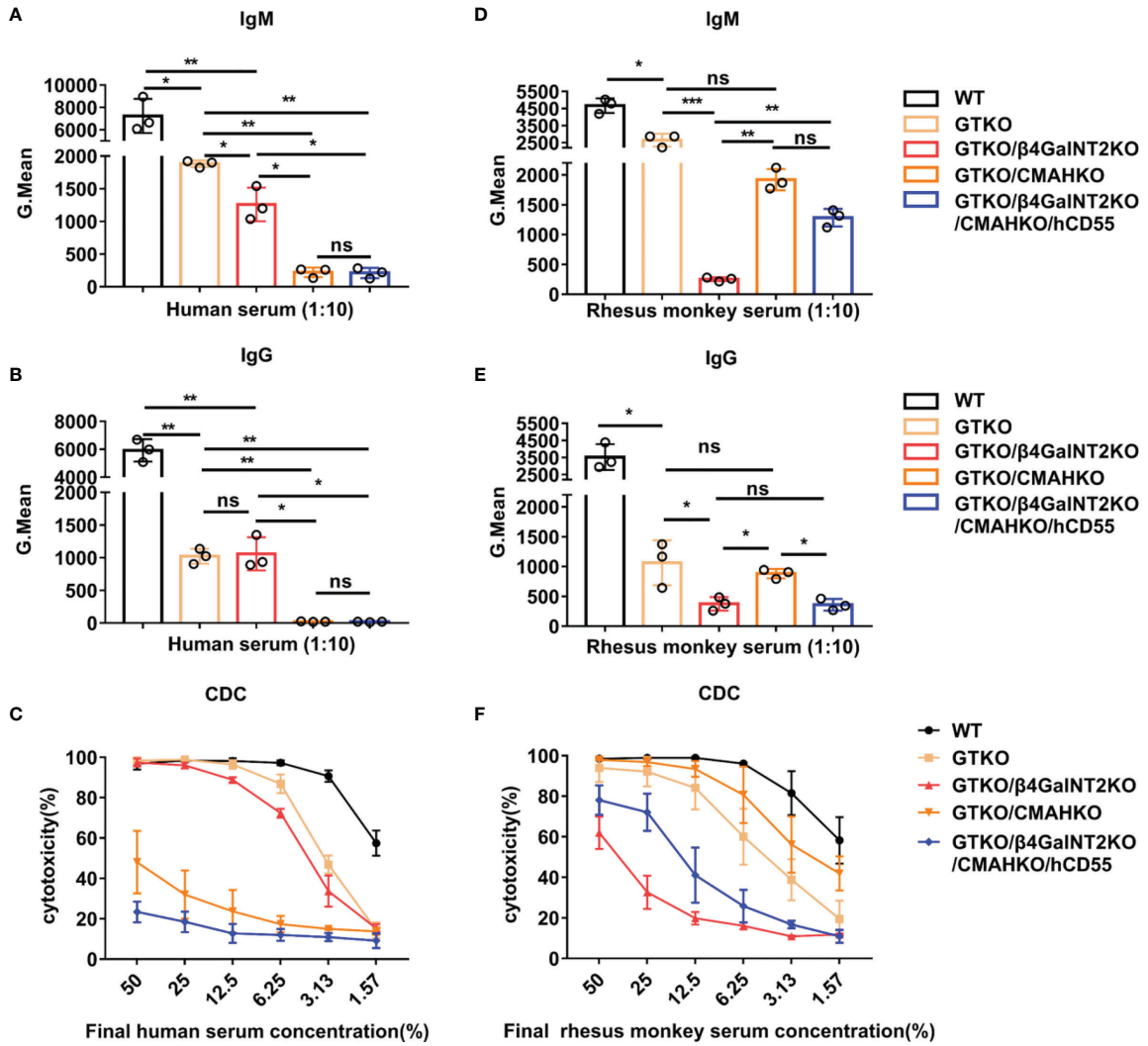

FIGURE 5 | The role of Sda and Neu5Gc expression in the binding of human vs. monkey serum antibody to GTKO pig PBMCs and in CDC against these cells. IgM/IgG antibody binding and CDC were measured by flow cytometry after co-culture of pooled human or monkey sera with PMBCs from WT, GTKO, GTKO/β4GalNT2KO, GTKO/CMAHKO, or TKO/hCD55 pigs. **(A, B, D, E)** Pooled human or rhesus monkey serum IgM/IgG binding (at a serum concentration of 10%) to pig PBMCs. **(C, F)** Pooled human or rhesus monkey serum CDC (at serum concentrations of 50% to 1.57%) against pig PBMCs. Each sample was measured three times. All data are presented as means ± SD (*P<0.05, **P< 0.01, ***P< 0.001; ns, non-significant). The P value comes from three independent experiments.

respectively), suggesting that anti-Sda antibodies accounted for more than half of the non-Gal-specific antibodies generated after transplantation (**Figure 6A**). In addition, we measured CDC against pig PBMCs using the four serum samples from the two monkeys before and after transplantation, and the results showed that the cytotoxicity of the same serum to GTKO/β4GalNT2KO pig PBMCs was lower than that against GTKO pig PBMCs, indicating that anti-Sda antibodies have cytotoxic activity against pig cells (**Figure 6B**).

DISCUSSION

Rhesus monkeys are often used in pig-to-NHP xenotransplantation studies (6, 12, 21, 22). There may be differences in natural anti-pig antibodies and immune reactivity to pig xenoantigens between rhesus monkeys and humans, so the genetically engineered pigs that favor long-term xenograft survival in rhesus monkeys may differ from those that are suitable for humans. In the present study, we clearly demonstrate through a series of *in vitro* experiments that, after Gal, Sda is the most important xenoantigen for pig-to-rhesus monkey xenotransplantation, whereas Neu5Gc is more important for pig-to-human xenotransplantation. In addition, we provide the first evidence that Sda can induce a significant antibody response in GTKO/hCD55 pig-to-rhesus monkey kidney xenotransplantation, and also that induced anti-Sda antibodies play an important role in the development of acute humoral xenograft rejection. These results suggest that a GTKO/β4GalNT2KO pig will be more

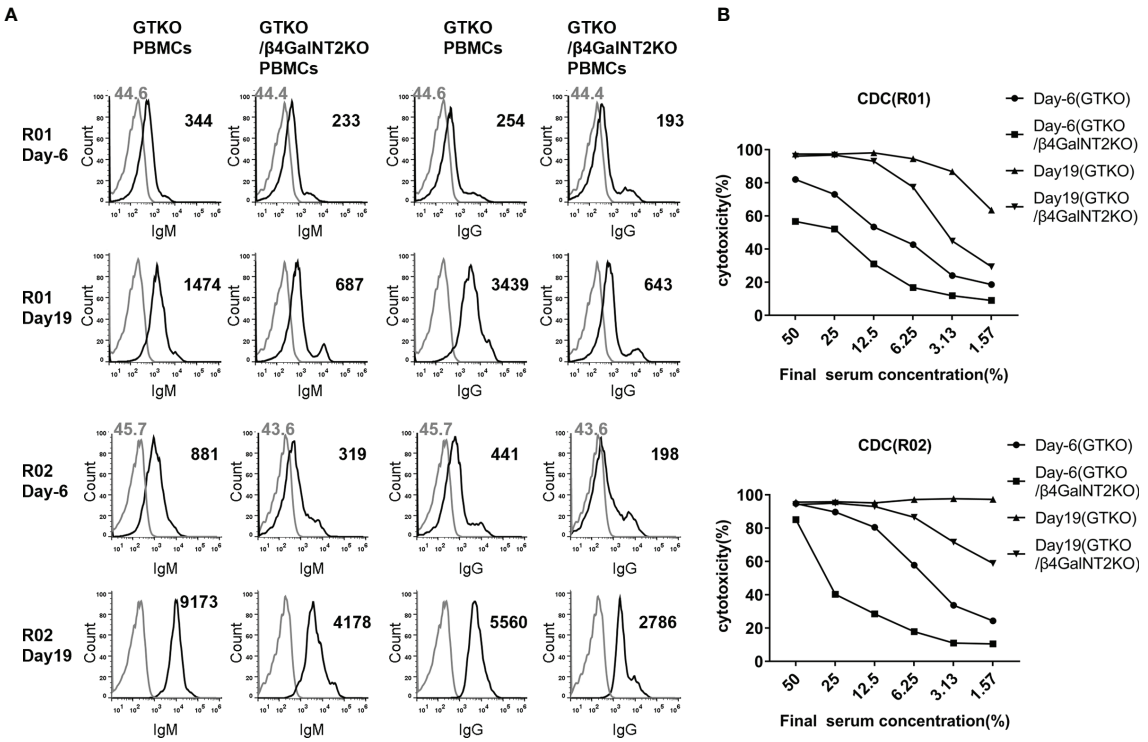

FIGURE 6 | The role of induced antibodies against Sda in the development of acute humoral xenograft rejection in GTKO/hCD55 pig-to-rhesus monkey kidney xenotransplantation. Renal xenotransplantation was previously performed in four rhesus monkeys using kidneys from GTKO/hCD55 pigs. Two of the four recipient monkeys (R01 and R02) generated high levels of circulating antibodies against non-Gal antigens and developed acute humoral xenograft rejection on day 19. Serum samples collected before transplantation (day -6) and at the time of rejection (day 19) were used to measure IgM/IgG antibody binding and CDC against GTKO (Sda^{mo}) or GTKO/β4GalNT2KO pig PBMCs. **(A)** The FACS results of serum IgM and IgG binding to pig PBMCs (at a serum concentration of 10%). Cells were incubated with medium served as negative controls (gray lines). The number represents the geometric mean fluorescence intensity (Gmean). **(B)** Comparison of CDC (at serum concentrations of 50% to 1.57%) of rhesus monkey (R01 and R02) pre-transplant (day -6) and post-transplant (day 19) serum against GTKO and GTKO/β4GalNT2KO pig PBMCs.

advantageous than the other strains tested for a pig-to-rhesus monkey xenotransplant model.

Most pig-to-NHP xenotransplantation studies to date have used GTKO pigs with or without the additional expression of certain human transgenic proteins (6, 16, 21–24). In these studies, animals with low levels of anti-pig antibodies and CDC were often selected as recipients for better graft survival (21, 22). However, few studies have measured the expression levels of xenoantigens other than Gal in donor pigs. Sda may be the most important xenoantigen for NHPs after Gal. In the present study, we have shown for the first time (to our knowledge) that the mean level of Sda expression in GTKO pigs is significantly higher than that in WT pigs, which may be related to the increased compensatory expression of other carbohydrate xenoantigens after Gal knockout. In addition, we found great differences in Sda expression level among individual GTKO pigs. These novel findings suggest that it is also necessary to assess Sda expression in donor pigs before GTKO pig-to-NHP xenotransplantation.

In 2015, Estrada JL et al. conducted *in vitro* studies using PBMCs from GTKO, GTKO/CMAHKO, and TKO pigs and found that inactivation of the β4GalNT2 gene reduces the binding of human and NHP antibody to pig cells (11). These results suggested that Sda expression in pigs may play a role in xenograft rejection. However, because of the lack of GTKO/β4GalNT2KO pigs in that study, the specific role of Sda antigen in xenotransplantation remains uncertain. In the last 3 years, two studies used rhesus monkey serum (n=14) and baboon serum (n=72), respectively, to perform *in vitro* antibody binding assays and CDC assays using PBMCs from GTKO, GTKO/β4GalNT2KO, or TKO pigs and found that the binding of both rhesus and baboon serum antibody and CDC against GTKO/β4GalNT2KO pig cells were significantly reduced (12, 17). In the current study, we have used GTKO pigs with different levels of Sda expression (high, moderate, low) and GTKO/β4GalNT2KO pigs to provide PBMCs and found that the overall binding of monkey serum IgM/IgG antibody and CDC against pig PBMCs decreased significantly as Sda expression was reduced. The clear dose-effect relationship that we observed confirms the important role of Sda in GTKO pig-to-rhesus monkey xenotransplantation. In addition, we found that the levels of monkey serum antibody binding and CDC against GTKO pig PBMCs with a low

expression of Sda were almost the same as those to GTKO/β4GalNT2KO pig PBMCs, suggesting that selecting GTKO pigs with low Sda expression as donors may achieve effects similar to those achieved by using GTKO/β4GalNT2KO pigs in a pig-to-rhesus xenotransplantation model.

We used pooled rhesus monkey sera and pooled human sera to perform *in vitro* antibody binding and CDC experiments with PBMCs from WT, GTKO, GTKO/B4GalNT2KO, GTKO/CMAHKO, and TKO pigs and demonstrated that Neu5Gc is important for pig-to-human xenotransplantation, whereas Sda is more important for pig-to-rhesus monkey xenotransplantation. These results are consistent with previous reports (11, 12, 15, 17) and indicate that preclinical xenotransplantation studies using NHPs do not fully mimic the immune response in humans after xenotransplantation. When conducting preclinical studies with rhesus monkeys or baboons, we need to consider using genetically edited pigs that are different from those suitable for humans.

Thus far, it has remained unclear whether the expression of Sda can induce a significant antibody response after GTKO pig-to-rhesus monkey xenotransplantation and subsequently contribute to the development of acute humoral xenograft rejection. Only one published paper has reported that there is positive antibody deposition in the rejected renal xenografts of long-term survivors and positive staining for the Sda antigen, suggesting that some of the late antibody-mediated injury may be directed against Sda (22). In the present study, we collected serum samples from two monkeys who had received GTKO pig kidney transplantation, which generated high levels of circulating non-Gal antibodies and developed acute humoral xenograft rejection. By measuring IgM/IgG antibody binding and CDC against GTKO or GTKO/β4GalNT2KO pig PBMCs, we found that anti-Sda antibodies accounted for more than half of the induced anti-non-Gal antibodies at the time of rejection and that anti-Sda antibodies showed significant cytotoxic activity against pig cells. To our knowledge, this is the first demonstration that the Sda antigen can induce significant antibody production and play an important role in mediating xenograft rejection in a pig-to-rhesus model. Since *in vivo* antibody binding is expected to occur first on endothelial cells of the graft in xenotransplantation, it is worth further attention whether the antibody binding results detected by donor PBMCs *in vitro* can reflect the "true danger" of the xenograft in the recipient.

In conclusion, we have demonstrated, for the first time that induced anti-Sda antibodies as well as natural anti-Sda antibodies play an important role in GTKO pig-to-rhesus monkey xenotransplantation, providing further evidence for GTKO/β4GalNT2KO pigs as the preferred organ source for rhesus monkeys used as recipients in a preclinical model of xenotransplantation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics committee of Tongji Hospital, Tongji Medicine College, Huazhong University of Science and Technology (TJ-C20181002). All human subjects were adults. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) at the Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (TJH-201905007).

AUTHOR CONTRIBUTIONS

GC and YW designed the experiments. HF, TL, JD, QX, and LW performed the *in vitro* experiments. GC, SC, and LZ performed the transplant surgery. DP and JD provided different genetically engineered pigs. HF, TL, JD, and GC analyzed the data and prepared the figures. HF wrote the article. GC critically revised the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.849711/full#supplementary-material

Supplementary Figure 1 | The role of Sda expression in the binding of monkey serum antibody to PBMCs from another group of GTKO pigs and in CDC against these cells. The IgM/IgG antibody binding and CDC were measured by flow cytometry after the co-culture of the pooled monkey sera with PMBCs from another group of GTKO pigs (Sda^{hi}, Sda^{mo}, and Sda^{lo}). **(A)** The expression of Gal and Sda on PBMCs from three GTKO pigs with different levels of Sda expression. **(B)** Flow cytometric histograms showing binding of rhesus monkey IgM/IgG antibody (top/middle row, serum diluted 1:10, Gmean values were shown) to pig PBMCs and CDC (bottom row, serum diluted 1:6, percentages were shown) against the same PBMCs.

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REVIEW
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Current Topics of Relevance to the Xenotransplantation of Free Pig Islets

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Pig islet xenotransplantation is a potential treatment for patients with type 1 diabetes. Current efforts are focused on identifying the optimal pig islet source and overcoming the immunological barrier. The optimal age of the pig donors remains controversial since both adult and neonatal pig islets have advantages. Isolation of adult islets using GMP grade collagenase has significantly improved the quantity and quality of adult islets, but neonatal islets can be isolated at a much lower cost. Certain culture media and coculture with mesenchymal stromal cells facilitate neonatal islet maturation and function. Genetic modification in pigs affords a promising strategy to prevent rejection. Deletion of expression of the three known carbohydrate xenoantigens (Gal, Neu5Gc, Sda) will certainly be beneficial in pig organ transplantation in humans, but this is not yet proven in islet transplantation, though the challenge of the '4th xenoantigen' may prove problematic in nonhuman primate models. Blockade of the CD40/CD154 costimulation pathway leads to long-term islet graft survival (of up to 965 days). Anti-CD40mAbs have already been applied in phase II clinical trials of islet *allo*transplantation. Fc region-modified anti-CD154mAbs successfully prevent the thrombotic complications reported previously. In this review, we discuss (I) the optimal age of the islet-source pig, (ii) progress in genetic modification of pigs, (iii) the immunosuppressive regimen for pig islet xenotransplantation, and (iv) the reduction in the instant blood-mediated inflammatory reaction.

Keywords: immunosuppression, islets, nonhuman primate, pig, genetically-engineered, type 1 diabetes, islet transplantation, xenotransplantation

Abbreviations: CMAH, cytidine monophospho-N-acetylneuraminic acid hydroxylase; Gal, galactose-α1,3-galactose; GTKO, 1,3-galactosyltransferase gene-knockout; IBMIR, instant blood-mediated inflammatory reaction; mAbs, monoclonal antibodies; Neu5Gc, N-glycolylneuraminic acid; NHP, nonhuman primate; NICC, neonatal islet cell clusters; PD-L1, programmed cell death ligand 1.

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INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disease characterized by pancreatic islet cell destruction by $CD4^+$ and $CD8^+$ T cells and autoantibodies, resulting in insulin deficiency and hyperglycemia (1). Conventional treatment of T1D includes exogenous insulin therapy, which reduces, but may not prevent, the development of the long-term complications of hyperglycemia. In late-stage T1D patients, especially those with 'brittle' diabetes, it is difficult to prevent complications such as cardiovascular disease, retinopathy, nephropathy, and life-threatening hypoglycemic episodes (1).

Islet *allo*transplantation has been identified as an efficient therapy for T1D, but, faced with the shortage of pancreases from deceased human donors, pig-to-human islet xenotransplantation has emerged as a potential alternative (2). Although pig-to-nonhuman primate (NHP) islet xenotransplantation has resulted in insulin independence, several problems remain.

The age of the islet-source pig may be important to islet quality. Adult pigs have a mature islet structure, lower galactose-α1,3-galactose (Gal) expression on islets, and a higher islet yield (3). Neonatal pig islets are easier to isolate and at a lower cost (3). To overcome immunological rejection of pig-to-NHP islet transplants, genetic modification of the source pig plays an important role by deleting xenoantigen expression and introducing human 'protective' proteins (4). New alternative modifications, e.g., expression of programmed cell death ligand 1 (PD-L1), are being explored. A consensus has been reached that, in regard to the transplantation of pig organs into humans, the expression of the three known carbohydrate xenoantigens (Gal, Neu5Gc, Sda) should be deleted (resulting in triple-knockout [TKO] pigs) (4, 5), but this remains uncertain after pig islet transplantation. However, there is a limitation in the TKO pig-to-NHP model because of the problem of the '4th xenoantigen'.

The selection of the immunosuppressive regimen plays a critical role in preventing the adaptive immune response (6). Although conventional immunosuppressive regimens are inefficient in preventing the adaptive response to pig cells, blockade of the CD40/CD154 costimulation pathway is successful, and has resulted in insulin-independence for a maximum of 965 days) (7). Emerging Fc region-modified anti-CD154mAbs successfully prevent the thrombotic complications seen previously (8, 9). Although anti-CD154 agents may be preferable, anti-CD40mAbs have already been applied in phase II clinical trials of human kidney *allo*transplantation (10).

In this review, we consider (i) the optimal age of the islet-source pig, (ii) the potential of genetic modification of the pig, (iii) the selection of the immunosuppressive regimen for pig-to-primate islet xenotransplantation, and (iv) potential steps to reduce the instant blood-mediated inflammatory reaction (IBMIR). We also briefly discuss the possible directions for future research.

DONOR AGE

Based on previous studies of pig-to-NHP islet xenotransplantation, pigs can be divided into three age groups: adult (>12 weeks), neonatal (~first 14 days after birth), and fetal. Their characteristics are summarized in **Table 1**. As fetal pig islets are not currently considered ideal sources for xenotransplantation due to defects in β-cell yield and immunogenicity, we will focus on adult and neonatal pigs.

Adult Pig Islets

To date, adult pig islets transplanted into NHPs have displayed the longest survival time (965 days) and have always been considered the primary source for islet xenotransplantation due to their superior islet yield, immediate insulin response, lower Gal expression, and higher β-cell percentage compared with neonatal pigs (**Table 1**). Female adult pigs that have produced >2 litters (retired breeders, usually >2 years old and > 200 kg) are preferred over young adult pigs because they consistently provide a higher yield of high-quality islets (3, 11). We add the ref: Bottino R, 2007 Our previous review summarized the above advantages (3). Using GMP-grade collagenase (collagenase AF-1 and liberase MTF C/T), one adult pig can yield up to 720,000IEQ (12), which is enough for islet

TABLE 1 | Characteristics of islets in pigs of different ages.

Characteristic	Fetal	Neonatal	Adult
Isolation procedure	Very simple (no purification)	Simple (No purification)	Difficult
Culture procedure	Resistance to hypoxia and inflammation	Resistance to hypoxia and inflammation	Difficult (Fragile), but not necessary
Early islet loss from IBMIR	Low (inflammation resistance)	Low (inflammation resistance)	Moderate (susceptible to inflammation)
Proliferation *in vivo*	Good	Good	Little
In vivo insulin production	Delay >2 months	Delay > 1 month	No delay
In vitro GSIS	Poor	Good	Good
Gal expression	High	High	Low
Islet yield (IEQs/pancrease)	~8, 000	25,000-64,000	200,000-720,000
Islet yield (IEQs/g)	NA	5,000-12,500	1,000-16,000
β-Cells % (after culture)	~10%	~25%	~70%
Risk of pathogen transmission	Extremely low	Low	Low
Islet isolation cost	NA	$0.02/IEQ	$0.09/IEQ
Cost	Low	Low	High

Gal, galactose-α1,3-galactose; GSIS, Glucose-stimulated insulin secretion; IBMIR, the instant blood-mediated inflammatory reaction; NA, not available.

xenotransplantation in a diabetic patient of approximately 60kg in weight. However, the limitations of adult pig islets include difficulty in isolation, higher costs for pig maintenance and islet isolation, and poor proliferative capacity (3) (**Table 1**).

Neonatal Islet Cell Clusters (NICC)

There have been only a few reports using NICC for transplantation into NHPs, with the longest survival being 260 days (13–16). The advantages of NICCs include (I) the need for only a short period of pig maintenance after birth, thus reducing the costs, (ii) easier isolation, thus increasing success and reducing isolation cost ($0.02/IEQ) compared to adult pig islets ($0.09/IEQ) (17), and (iii) greater proliferative capacity (3). However, there are some limitations. First, NICCs must be cultured to reaggregate the islet cluster before transplantation, although various modified culture media, the addition of growth factors, and coculture with mesenchymal stromal cells facilitate NICC islet maturation and function (18–21). Second, there is a delay in the *in vivo* response to glucose after transplantation (that may be >4 weeks in NHPs), and so measuring islet loss is difficult (3).

The difference in the glucose-stimulated insulin secretion index between adult pig islets and NICC remains controversial. Some research has indicated that NICC has a significantly higher stimulation index (4.7± 0.58) than adult pig islets (1.75 ± 0.60) (17), but other studies show the opposite (summarized in **Table 2**) (12, 17, 21–23). Therefore, the glucose-stimulated insulin secretion of adult pig islets and NICC may be equivalent.

In summary, a consensus on the optimal age for pig islet xenotransplantation has not been reached. Adult pig islets should be the primary option as better results have been achieved following transplantation into NHPs, but NICCs are regarded as a promising alternative islet source with several significant superiorities.

GENE MODIFICATION

The development of CRISPR/Cas9, an efficient genome editing technique, provides the capacity to produce pigs with multiple genetic modifications for xenotransplantation (**Table 3**) (24–41). We will here mainly focus on gene modification targets for carbohydrate xenoantigens and cellular immune response-related genes.

Carbohydrate Xenoantigen Genes

A consensus has been reached that the three known carbohydrate xenoantigen genes (Gal, Neu5Gc, Sda) should be knocked-out for pig-to-human organ transplantation (**Table 4**), but this is not ideal for pig-to-NHP organ transplantation because of the problems associated with the '4th xenoantigen' (discussed in 42–46). It is well-known that pig organ grafts from CMAHKO pigs are associated with increased NHP IgM and IgG binding and serum complement-mediated cytotoxicity, resulting in acute xenograft rejection (42–46).

To our knowledge, the transplantation of TKO pig islets into NHPs has not been reported, and it remains unknown whether the '4th xenoantigen' is exposed in TKO pig islets as it is in vascular endothelial cells. Whether TKO islets would provide an advantage in this regard remains uncertain.

Of relevance to this point, there were no statistically significant differences in human IgM and IgG binding to isolated islet cells from GTKO/hCD46 and GTKO/hCD46/NeuGcKO pigs (47). Knockout of CMAH may therefore possibly have a different effect in islets than in solid organs. In one report, GTKO/CMAHKO pigs developed pathological features that are similar to those seen in anemia, possibly associated with variations in glycosylation on the red blood cell membranes of these pigs (48). Obukhova et al. have reviewed CMAH comprehensively (49).

If neonatal pigs are the source of islets (i.e., NICCs), in which expression of Gal is considerable, the deletion of expression of Gal (and possibly of Neu5Gc and Sda) will be advantageous.

Differences in N- and O-glycan profiles between human and porcine islets might prove to be the next gene modification sites. Novel xenoantigens include complex-type N-glycans with terminal neuraminic acid residues and high-mannose-type N-glycans with core fucosylation (50). Carbohydrate antigen microarrays in pigs and cynomolgus monkeys have revealed natural non-αGal antigens (e.g., Tn antigen, T antigen, GM2 glycolipid) and novel carbohydrate structures (e.g., Galβ1-4GlcNAcβ1-3Galβ1 and N-linked glycans with Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ) that are responsible for the IgM and IgG anti-carbohydrate antibody responses (51, 52). These findings suggest future gene modification sites to eliminate anti-carbohydrate antibody responses in pig-to-primate islet xenotransplantation.

For future studies of the 4th xenoantigen(s), several sources might be helpful, e.g., the database of Glycomics (http://www.

TABLE 2 | *In vitro* stimulation index of neonatal and adult pig islets.

Reference	Neonatal	Adult	Digestion Enzyme
Vanderschelden et al. (17)	4.7 + 0.58	1.75 + 0.60	Sigma Type V Collagenase
Smith et al. (22)	1.8 ± 0.3	8.5 ± 1.2	Liberase HI
Emamaullee et al. (23)	1.78 ± 0.14	NA	Collagenase
Hassouna et al. (21)	1.7 ± 0.2	NA	Collagenase
Kwak et al. (12)	NA	2.07 ± 0.02	Collagenase P
Kwak et al. (12)	NA	4.73 ± 0.23	Collagenase AF-1*
Kwak et al. (12)	NA	3.87 ± 0.12	Liberase MTF C/T*

GMP grade; NA, not available.

TABLE 3 | Selected gene modifications in pigs of relevance to pig-to-NHP islet xenotransplantation.

Purpose	Modified genes	Ref
Deletion of carbohydrate xeno-antigens	α1,3-galactosyltransferase gene knockout (GTKO)	(24, 25)
	Cytidine monophospho-N-acetylneuraminic acid hydroxylase gene knockout (CMAHKO)	(26)
	β-1,4-N-acetylgalactosaminyltransferase-2 gene knockout (β4GalNT2)	(27)
Prevention of inflammation	Human hemagglutinin-tagged-human hemeoxygenase-1 gene knock-in (HO-1)	(28)
	Soluble human tumor necrosis factor receptor I IgG1-Fc gene knock-in (shTNFRI-Fc)	(28)
Prevention of complement-mediated injury	CD46 gene knock-in	(29)
	CD55 gene knock-in	(30)
	CD59 gene knock-in	(31)
Prevention of coagulation dysfunction	Human thrombomodulin gene knock-in (hTBM)	(32)
	Human endothelial protein C receptor gene knock-in (EPCR)	(33)
	Human tissue factor pathway inhibitor-2 knock-in (hTFPI)	(34)
	CD39 gene knock-in	(35)
Protection against cellular immune response	Cytotoxic T-lymphocyte antigen-4 immunoglobulin (CTLA4-Ig) or LEA29Y transgene (CTLA4-Ig mutation)	(36)
	MHC class II transactivator knockdown (CIITA-DN)	(37)
	β2-microglobulin knock-out (B2MKO)	(38)
	CD47 gene knock-in	(39)
	Programmed cell death ligand 1 gene knock-in (PD-L1)	(40)

functionalglycomics.org/). The National Center for Functional Glycomics (NCFG) (https://ncfg.hms.harvard.edu/) offers a CFG mammalian-type glycan microarray, with 600 glycans present, that might be helpful in studying xenoantigens in the future.

Cellular Immune Response-Related Genes

Progress in gene modification aimed at protecting xenografts from the adaptive immune response has been made recently. For example, knock-in of CTLA4-Ig or the high-affinity variant LEA29Y (36, 53), knockout or knockdown swine leukocyte antigen (SLA) class I and class II (37, 54), and *in vitro* tests on SLA class I and class II-silenced cells have reported significantly reduced xenogeneic T cell and natural killer cell responses, and antibody-mediated cell-dependent responses to islet cell clusters (55). However, CTLA4-Ig or LEA29Y transgenic pigs face the problems of hypoimmunity (36, 56).

Immune checkpoint blockade is a promising approach to control pathogenic immune responses. Immunomodulation with PD-L1 improves islet allotransplantation outcomes (57–63), and may facilitate successful xenotransplantation. PD-L1 is a ligand that reduces the proliferation and activation of T cells, B cells, and monocytes through interaction with PD-L1 receptors on these cells, and prevents cell-mediated lysis from $CD8^+$ T cells by reducing their proliferation and cytokine secretion (40). Programmed cell death protein 1 blockade has successfully achieved clinical objectives in the treatment of cancer (64–66). In xenotransplantation, pigs transgenic for PD-L1 have been successfully generated, and cells from these pigs prevent human T cell cytotoxicity and B cell activation *in vitro* (57, 58), with similar results in a pig-to-rat xenotransplantation model (67). In contrast, islet PD-L1 deficiency has been associated with increased allograft rejection and increased inflammatory cell infiltration (68). Testing of the transplantation of pig islets expressing PD-L1 in NHPs should be a future research direction.

In summary, whether the 4th xenoantigen is exposed in islets after CMAHKO remains uncertain, and more research on the cellular response (that will be the next obstacle to explore) is required (69).

IMMUNOSUPPRESSIVE REGIMEN

The main objective of the immunosuppressive regimen is to inhibit T cell activation and prevent subsequent T cell-dependent dendritic cell activation and activation of B cells and macrophages. Immunosuppressive regimens based on conventional (FDA-approved) therapy have proved inadequate, although islet graft survival of 222 days has been reported (70). In contrast, blockade of the CD40/CD154 costimulation pathway has resulted in maximal islet graft survival of 965 days (**Table 5**) (7, 13, 70–76). The major mechanistic effects, advantages, and side-effects of the key immunosuppressive agents of relevance to xenotransplantation have been reviewed by Bikhet and his colleagues (77). Samy et al. have reviewed the role of costimulation pathway blockade in xenotransplantation (78). Here we will focus on novel immunosuppressive regimens based on blockade of the CD40/CD154 costimulation pathway.

TABLE 4 | Known carbohydrate xenoantigens expressed on pig cells.

Carbohydrate (Abbreviation)	Responsible enzyme	Gene-knockout pig
1.Galactose-α1,3-galactose (Gal)	α1,3-galactosyltransferase	GTKO
2.N-glycolylneuraminic acid (Neu5Gc)	CMAH	CMAH-KO
3.Sd[a]	β-1,4N-acetylgalactosaminyltransferase.	β4GalNT2-KO

CMAH, Cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH).

TABLE 5 | Immunosuppressive protocols associated with prolonged periods of insulin-independence and islet graft survival.

Major agent	Islet-source pig	Immunomodulatory regimen	Maximum Insulin independence	Maximum graft survival	Ref
Anti-CD154	WT (adult)	Anti-ICAM-1 mAbs (MD-3), anti-CD154 mAbs (5C8), Sirolimus, TNF-a-neutralizing mAb (adalimumab), Anakinra, Ganciclovir, Clopidogrel, Heparin	520d	520d	(71)
	WT (adult)	ATG, anti-CD154 mAbs (5C8), Sirolimus, CVF, TNF-a-neutralizing mAb (adalimumab)	603d	603d	(72)
	hCD46 (adult)	ATG, Anti-CD154 mAbs (ABI7953), MMF, Dextran sulfate, Prostacyclin, Methylprednisolone, Aspirin, Ganciclovir, Famotidine, Heparin	365d	365d	(73)
	GTKO, hCD46, hCD39, hTFPI (adult)	ATG, Anti-CD154 mAbs (h5c8), MMF, Dextran sulfate, Prostacyclin, Methylprednisolone, Aspirin, Ganciclovir, Famotidine, Heparin	365d	365d	(73)
	hCD46 (adult)	ATG, Anti-CD154 mAb (ABI7953), Dextran sulfate, Methylprednisolone, Aspirin, Prostacyclin	396d	396d	(74)
Anti-CD40	WT (neonatal)	Anti-CD40 mAbs (Chi220), aIL-2R (Basiliximab), Belatacept, Sirolimus	203d	>203d	(13)
	WT (adult)	Anti-CD40 mAbs (2C10R4), Sirolimus, ATG, CVF, Tacrolimus, Adalimumab, Methylprednisolone	266d	320d	(75)
Anti-CD154 plus Anti-CD40	WT (adult)	ATG, CVF, anti-CD154 mAbs (5C8), Anti-CD40 mAbs (2C10R4), Sirolimus, TNF-a-neutralizing mAb (adalimumab), Treg	965d	965d	(7)
Conventional	WT (adult)	ATG, Rituximab, Belimumab, Sirolimus, Tacrolimus, Tofacitinib, Adalimumab, Anakinra, CVF, IVIg	130d	201d	(76)
	WT (adult)	ATG, Belimumab, Sirolimus, Tacrolimus, Abatacept, Tofacitinib, Adalimumab, Anakinra, Tocilizumab, IVIg, Aspirin	90d	222d	(70)

aIL-2R, IL-2 receptor-specific antibody; ATG, anti-thymocyte globulin; CVF, cobra venom factor; GTKO, 1,3-galactosyltransferase gene-knockout; hTFPI, knock-in human tissue factor pathway inhibitor-2; ICAM-1, intercellular cell adhesion molecule-1; IVIg, intravenous immunoglobulin; mAbs, monoclonal antibodies; MMF, mycophenolate mofetil; Treg, regulatory T cell; WT, wild type.

Immunosuppressive Regimens Based on Anti-CD40mAbs

Anti-CD40mAbs are a chimeric form of Fab combined with IgG Fc fragments to prevent the stimulation of B and T cells through blockade of the CD40/CD154 pathway, which also participates in regulating thrombosis, tissue inflammation, and hematopoiesis (79). Unlike anti-CD154mAbs, no significant thrombogenic complications have been observed in anti-CD40mAb studies (10). Islet graft survivals are summarized in **Table 5**.

To date, some anti-CD40mAbs have completed phase II clinical trials of allotransplantation (but *not* in islet transplantation). These included bleselumab (ASKP1240), iscalimab (CFZ533), and BI 655064 (10, 80, 81). Among them, ASKP1240 demonstrated good results with a favorable benefit-risk ratio and no thromboembolic events in a phase II clinical kidney transplantation trial (10). Treatment with 2C10R4 was associated with the longest pig islet graft survival in NHPs to date (maximum insulin-independence 950 days, maximum graft survival 965 days) (7). However, anti-CD40mAbs may be associated with adverse effects, e.g., a temporary increase in liver enzymes (ASKP1240) (82, 83), significant depletion of peripheral blood B cells (Chi220) (13), and inhibition of T regulatory cell (Treg) expansion (2C10R4) (84).

Immunosuppressive Regimens Based on Anti-CD154 Agents

Anti-CD154 agents also provide efficient CD40/CD154 pathway blockade (85), but were originally associated with thromboembolic complications (BG9588, hu5c8, IDEC-131, ABI793) (86–88), although the situation with IDEC-131 remains controversial (89). They were demonstrated to be preferable to anti-C40mAbs in pig islet transplantation in NHPs (**Table 5**) (72, 75). Modifications to the Fc region on CD154 agents, the binding site for the Fc receptor (FcgRIIA) on platelets (85), appear to have eliminated thromboembolic events (e.g., CDP7657 and BMS-986004 in rhesus macaques, and MEDI4920 in cynomolgus monkeys) (77). To date, CDP7657, BMS-986004, and MEDI4920 have completed phase I or II clinical trials (*not* in islet transplantation) without obvious complications (8, 9, 90–92).

Overall, although anti-CD40mAbs have proved successful in pig-to-NHP islet xenotransplantation, the new anti-CD154 agents may prove preferable for clinical trials (**Table 6**) (9, 10, 75, 80–83, 90, 92–102). Of importance, ongoing studies at the Massachusetts General Hospital indicate that *monotherapy* with an anti-CD154mAb (with *no* additional immunosuppressive therapy) prevents rejection of heterotopic heart and life-supporting kidney *allo*grafts in monkeys (Robin Pierson and Tatsuo Kawai, personal communications). This regimen, or a modification of it, has not yet been tested in xenograft models

Bikhet et al. published an immunosuppressive regimen that has proved moderately successful in pig solid organ transplantation in NHPs (77), but such a regimen may be too intensive to warrant use in patients with islet xenografts.

THE INSTANT BLOOD-MEDIATED INFLAMMATORY REACTION (IBMIR)

After infusion of islets into the portal vein (the preferred site at present), a substantial percentage of islets are lost in the immediate post-transplant period through an inflammatory response termed IBMIR. The loss is significantly greater if the islets are xeno-islets, e.g., pig islets into NHPs and pig islets to human blood *in vitro*

TABLE 6 | Agents that block the CD40/CD154 costimulation pathway that are currently in clinical trials and preclinical studies, an update of Bikhet 2021 (58).

Drug and company	Clinical trials	Results
Anti-CD40		
Bleselumab	Phase Ia/Ib:	
(ASKP124/4D11)	NCT01279538 (60, 72)	Well-tolerated in healthy humans and in kidney transplant recipients
Astellas	Phase II:	
	NCT01780844 (9)	well tolerated in kidney transplant recipients
	NCT01585233 (78)	well tolerated in moderate-to-severe plaque psoriasis patients
	NCT02921789	Kidney transplantation (without results)
Iscalimab	Phase I:	
(CFZ533)	NCT02089087 (73)	well tolerated in Rheumatoid Arthritis
Novartis	Phase I/II:	
	NCT02217410 (74, 75)	well tolerated in kidney transplant recipients
	Phase II:	
	NCT02291029 (76)	Has therapeutic potential in primary Sjogren's syndrome patients
	NCT02713256 (58)	Has therapeutic potential in Graves' disease patients
	NCT02565576	Has therapeutic potential in Severe Myasthenia Gravis
	NCT03663335	Kidney transplantation (without results)
	NCT03781414	Liver transplantation (without results)
	NCT03610516	Lupus nephritis (without results)
	NCT03905525	Sjogren's syndrome (without results)
	NCT04129528	Type 1 Diabetes (without results)
	NCT03656562	SLE (without results)
BI 655064	Phase I:	
Boehringer Ingelheim	NCT01751776 (77)	Well-tolerated in healthy humans
	Phase II:	
	NCT01751776 (59)	Safety in rheumatoid arthritis patients with inadequate response to methotrexate
	NCT03385564	Lupus nephritis (without results)
	NCT02770170 (78)	Lupus nephritis (did not meet its primary CRR endpoint)
KPL-404	Phase I:	
Kiniksa	NCT04497662 (79)	Well-tolerated in healthy humans
2C10R4	Preclinical study (55, 80, 81)	Prolonged graft survival in pig-to-NHP cardiac and islet xenotransplantation, NHP islet allotransplantation
NIH NHP Resource Center		
Anti-CD154		
Dapirolizumab	Phase I:	
(CDP7657)	NCT01093911 (69)	Well tolerated in healthy humans and in patients with SLE
UCB AND BIOGEN	NCT01764594 (8)	Safety and efficacy in SLE patients
	NCT04571424	Healthy human (without results)
	Phase II:	
	NCT02804763 (71)	Well tolerated in healthy human and SLE. Has therapeutic potential in SLE
	Phase III:	
	NCT04294667	SLE (without results)
	NCT04976322	SLE (without results)
Letolizumab	Phase I/II:	
(BMS-986004)	NCT02273960	Safety in Immune thrombocytopenic purpura (ITP)
BMS	Phase I/II:	
	NCT03605927	Graft-versus-host disease (GVHD) (without results)
VIB4920	Phase I:	
(MEDI4920)	NCT02780388	Well tolerated in patients with rheumatoid arthritis
VielaBio	NCT02151110	Well tolerated in healthy adults
	Phase II:	
	NCT04046549	Kidney transplantation (without results)
	NCT04129164	Sjogren's syndrome (without results)
	NCT04163991	Rheumatoid arthritis (without results)
	NCT04174677	Kidney Transplantation (without results)

GVHD, graft-vs-host disease; ITP. immune thrombocytopenic purpura; mAb, monoclobal antibody; PEG, polyethylene glycol; SLE, systemic lupus erythematosus; TCP, thrombocytopenic purpura; NA, not available.

(103–107). Coagulation, platelet aggregation, complement activation, and neutrophil and monocyte infiltration play roles in this reaction (108). Several approaches to reduce the loss of islets have been explored, e.g., anticoagulation, complement depletion (109), and modified islet culture medium (110), but none has been entirely successful yet. The transplantation of islets from pigs with one or multiple genetic modifications may help protect the islets from early injury and loss (14, 74, 111–115). Moreover, alternative transplantation sites in intrapleural space greatly reduced IBMIR (116).

It is beneficial to add heparin or dextran sulfate to the peri-transplant regimen for their anticoagulant and complement-

modulating properties that reduce islet loss from IBMIR (109, 117–120). Low molecular dextran sulfate at low doses demonstrated good results in the prevention of IBMIR in phase II clinical islet *allo*transplantation study (NCT00789308) (119). Nanoparticle-based techniques improve the therapeutic efficacy of heparin. For example, polymeric nanocoating islets with heparin-polyethylene glycol (PEG) or chondroitin sulfate-PEG in an NHP islet allotransplant model was associated with significantly longer islet survival with reduced loss to IBMIR compared with PEG and naked islets (121, 122). Conjugated nanoparticles (heparin-immobilized superparamagnetic iron oxide) conjugated onto the surface of the islets attenuated IBMIR in a rat-to-mouse islet xenotransplantation model (123). Islet-surface modifications with streptavidin-CD47 protein, a chimeric construct expressing CD47 on the extracellular domain, efficiently prevent islet loss from IBMIR (124).

Cibinetide (Araim Pharmaceuticals Inc., Tarrytown, NY, USA) (a non-hematopoietic erythropoietin analogue) also showed islet-protective effects by reducing IBMIR-induced platelet consumption (125). Based on these studies, agents that reduce IBMIR, combined with the transplantation of islets from genetically-engineered pigs (e.g., pigs not expressing the known carbohydrate xenoantigens, but expressing human complement- and coagulation-regulatory proteins), and an optimal immunosuppressive regimen may increase graft survival and the therapeutic efficacy of islet xenotransplantation.

COMMENT

Key factors in successfully developing pig islet xenotransplantation include determination of the optimal age of the islet-source pig (adult or neonatal), the optimal genetic modifications that should be made to the pig, and the optimal immunosuppressive regimen that should be administered to the recipient. Whether the '4th' xenoantigen is problematic in the pig-to-NHP islet transplantation model needs to be clarified. More attention needs to be directed to genetic modifications that might reduce the instant blood-mediated inflammatory reaction and/or the adaptive immune response to pig islets. The advantages and disadvantages of immunosuppressive regimens based on anti-CD40 and anti-CD154 agents require clarification. Since the first case of successful pig-to-human kidney and heart transplantation had been reported recently (126, 127), we anticipate that pig islet xenotransplantation will become clinically successful when these remaining questions have been resolved.

AUTHOR CONTRIBUTIONS

LM, DC, and ZP initiated the review. LM and GS wrote the manuscript. DC, YL, JC, SZ, JD, YH, YN, YZ, and ZC revised the manuscript. All authors contributed to the article and approved the submitted version.

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Nanotechnology in Kidney and Islet Transplantation: An Ongoing, Promising Field

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Organ transplantation has evolved rapidly in recent years as a reliable option for patients with end-stage organ failure. However, organ shortage, surgical risks, acute and chronic rejection reactions and long-term immunosuppressive drug applications and their inevitable side effects remain extremely challenging problems. The application of nanotechnology in medicine has proven highly successful and has unique advantages for diagnosing and treating diseases compared to conventional methods. The combination of nanotechnology and transplantation brings a new direction of thinking to transplantation medicine. In this article, we provide an overview of the application and progress of nanotechnology in kidney and islet transplantation, including nanotechnology for renal pre-transplantation preservation, artificial biological islets, organ imaging and drug delivery.

Keywords: nanotechnology, kidney, islet, transplantation, promising field

INTRODUCTION

As a reliable survival choice for patients with end-stage organ failure, organ transplantation has progressed markedly in the era of modern medicine. With progress in surgical approaches, anesthesia, nursing and the addition of immunosuppressants, the survival of grafts has markedly improved. However, with the extension of human life expectancy and the increasing incidence of chronic diseases, the demand rate for organ transplantation may also increase, which will further aggravate the serious imbalance between organ demand and supply, the most important issue in transplantation medicine (1–3).

In addition to organ shortages, other challenges, such as the risk of transplantation, immune rejection, long-term immunosuppressants and their inevitable side effects, will limit the application of transplantation technology (2, 4–6). Fortunately, the combination of emerging technologies with transplantation approaches is influencing traditional transplantation methods (7), such as single-cell sequencing technology being used to determine cell heterogeneity and new molecular

characteristics for immune rejection and CRISPR/cas9 gene-editing technology being used to generate transgenic pigs to resolve issues of organ shortage (8, 9).

Nanotechnology has developed rapidly in the past few decades and has its applications in many fields, including medicine. Nanomaterials are usually divided into metallic, organic and semiconducting particles, with sizes ranging from 1 to 100 nm. The main purpose of nano medicine is to diagnose and treat diseases at the nano level using the properties and properties of nano materials (10). For example, nanomaterials are used as carriers to achieve drug targeting and controlled release (5, 10), improve pharmacokinetics (11) and reduce drug toxicity and side effects (12, 13). They have also been used for direct tumor treatment (14) and molecular imaging (15). Nanomedicine has unlimited potential. Its application in transplantation medicine overcomes the obstacles of many traditional transplantation methods, such as optimizing the pharmacokinetics of immunosuppressive drugs, improving organ or tissue preservation, aiding in the generation of artificial biological organs and improving organ imaging (5, 16–18).

We focused on the outcomes of combining nanotechnology with kidney transplantation and islet transplantation and summarized the current popular applications and latest progress in this field.

NANOTECHNOLOGY IN KIDNEY PRESERVATION

Implantable Artificial Kidney (IAK)

Kidney transplantation is the most promising way to cure renal failure, and dialysis is one way to extend the survival of patients who must wait because of the scarcity of kidney sources. However, dialysis inevitably transforms the daily lives of patients.

The combination of silicon nanotechnology and tissue engineering has given birth to a device that has changed the current situation: the IAK. The HemoCartridge of the IAK is responsible for filtration, and the IAK also possesses a bioreactor to culture renal tubular cell epithelium (BioCartridge). Benefiting from silicon nanotechnology, each microchip in the HemoCartridge forms a filtration channel resembling a glomerular slit diaphragm that can block molecules such as albumin and allow waste filtration of small molecules. The device, which is implanted surgically, uses blood pressure for filtering power, not relying on an electric pump and dialysate, and simulates the natural kidney function (**Figure 1**). This technology is of great significance for patients with chronic kidney disease. The implantation of an artificial kidney can greatly reduce the urgent need for renal transplantation and alleviate the current pressure of renal scarcity (19–24).

Donated After Circulatory Death (DCD) Kidney and Its Role in Alleviating Kidney Donor Deficiency

An additional way to alleviate the pressure associated with the kidney transplant donor shortage is the acceptance of less-ideal organs, such as the gradually increasing number of kidneys that are donated after circulatory death (DCD). The issue with DCD kidneys is their longer warm ischemic time, leading to injury. The effects of such injury are diverse. The kidney is particularly prone to acute tubular necrosis, resulting in a delay in the renal function recovery, while the pancreas is more prone to graft thrombosis and islet loss (25–27). However, these should not be reasons for abandoning DCD organs. The experience with the UK DCD kidney transplantation program supports the utilization of DCD organs to help alleviate the global shortage of kidneys from deceased donors (17, 28).

Suitability of Hypothermic Machine Perfusion (HMP) Over Static Cold Storage (SCS) for the Preservation of DCD Kidneys

Organ preservation is an important step before transplantation. At present, the main goal is to preserve the function of organs after they are removed from the body and reduce the injury of ischemia and reperfusion (29). SCS, which is easy to operate and inexpensive, has become the main method of organ preservation in solid organ transplantation. However, in the era of the increasing use of DCD organs, the preservation effect has been unsatisfactory.

Organ damage due to hypoxia as well as reperfusion is particularly pronounced in DCD organs. This has necessitated revisiting organ preservation methods that predate SCS, with a renewed emphasis on organ preservation to reduce organ damage from ischemia-reperfusion injury(IRI). A prospective study on HMP carried out by Moers et al. pulled the prelude to the use of mechanical perfusion for clinical organ preservation. Their team randomly divided the kidneys of 336 deceased donors into HMP and SCS groups and then transplanted the kidneys

Abbreviations: IAK, implantable artificial kidney; DCD, donated after circulatory death; HMP, hypothermic machine perfusion; SCS, static cold storage; NMP, normothermic machine perfusion; APACs, antiplatelet and anticoagulants; IRI, ischemia-reperfusion injury; EVLP, *ex vivo* lung perfusion; ECs, endothelial cells; TLN, thrombalexin; cRGD, cyclic RGD; EVs, extracellular vesicles; TRAMs, targeted rapamycin micelles; ISAs, immunosuppressive agents; ROS, reactive oxygen species; PTX, pentoxifylline; SPIONs, superparamagnetic iron oxide nanoparticles; LBL, layer-by-layer; PEG8, 8-arm-PEG-catechol; UFH-NHS, N-hydroxysuccinimidyl-linked unfractionated heparin; HNSIs, heparin nano-shielded islets; JAG-1, Jagged-1; CONPs, cerium oxide nanoparticles; MLBL, multi-layer-by-layer; CLBL, covalently-stabilized LBL; PERV, porcine endogenous retrovirus; NPCCs, neonatal porcine islet-like cell clusters; CR-NCS, cold-responsive nanocapsules; CAH, calcium alginate hydrogel; mTOR, mammalian target of rapamycin; PEG-PCL, poly(ethylene glycol)-b-poly (ϵ-caprolactone); PLGA, Poly (lactic-co-glycolic acid); PEG-PLGA, poly (ethylene glycol)- Poly (lactic-co-glycolic acid); HDLs, high density lipoproteins; ICAM-1, intracellular adhesion molecule-1; TCR, T cell receptor; APC, antigen presenting cells; PD-L1, programmed cell death-Ligand 1; PLG, poly (D,L-lactide-co-glycolide); NOD, non-obese diabetic; MP, microparticle; T1DM, type 1 diabetes mellitus; NHP, non-human primates; MN, magnetic nanoparticles; MHC, major histocompatibility complex; MRI, magnetic resonance imaging; SPIO, superparamagnetic iron oxide; IBMIR, immediate blood-mediated inflammatory response; USPIO, ultra-miniature superparamagnetic iron oxide; FeMRA, ferumoxytol-enhanced magnetic resonance angiography; FDA, food and drug administration; PTEN, phosphatase and tensin homologue; MPI, magnetic particle imaging; BLI, bioluminescence Imaging; FI, fluorescence Imaging; PFOB, perfluorooctylbromide; PFPE, perfluoropolyethers.

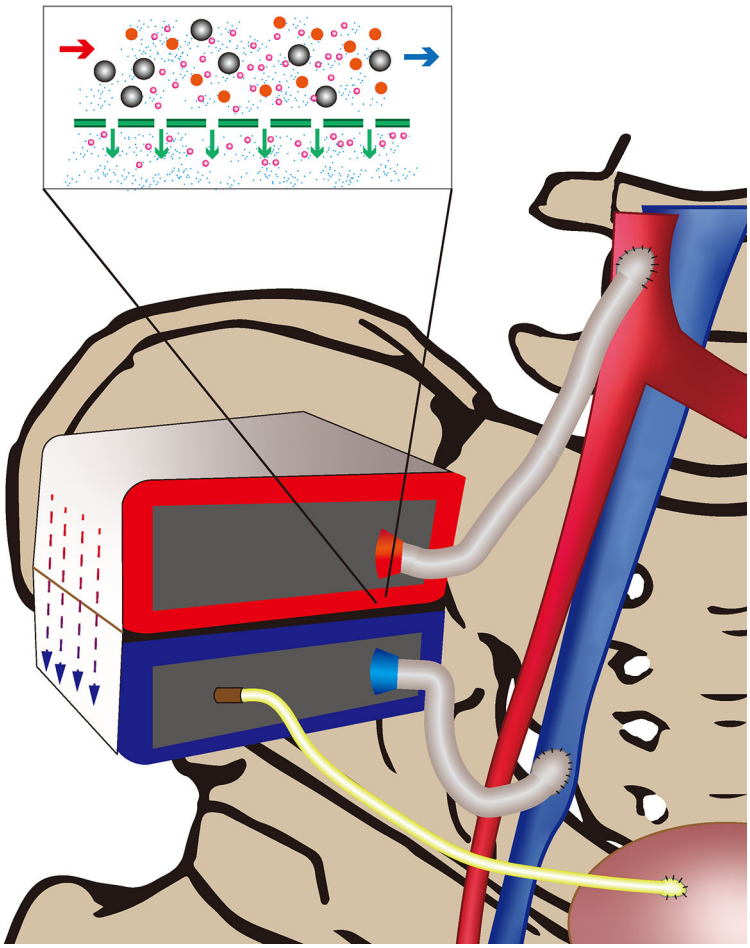

FIGURE 1 | Arterial blood (red) is filtered through the lak and the generated urine passes through a tubing (yellow) into the bladder. Filtered blood flows back into the vein (blue).

into 672 subjects. After one year of follow-up, it was found that perfusion reduced the incidence and extended the duration of a delayed graft function. The one-year graft survival was also better than that in the SCS group (30).

Normothermic Machine Perfusion (NMP) as an Alternative to Machine Perfusion

Since HMP does not completely mimic the organ circulatory environment in a physiological state, the effect of cold ischemia on graft injury as well as adverse graft outcomes has been noted. Therefore, NMP, which lacks the disadvantages of cold ischemia, has attracted widespread interest (31).

Arnaud et al. performed orthotopic transplantation after NMP of isolated rabbit kidney, suggesting that this method for organ preservation may be ideal for improving the current state of DCD organ preservation (32). Not only do normothermic perfused kidneys suffer less endothelial cell damage, but the partial damage induced by cold ischemia can be reversed, and the metabolic function of the organ can be restored as well, thereby improving the final transplantation outcome (33–35). Given the good results obtained in animal trials, NMP has been used in clinical trials with marginal kidneys, further demonstrating the superiority of this approach (36, 37).

The exploration of the utility of NMP continues. After Kaths et al. found that prolonged NMP in isolated porcine kidneys resulted in better graft outcomes than either SCS alone or that combined with short-term NMP (38). AnnemarieWeissenbacher's group were further explored in human kidney with developing an automated normothermic perfusion system. This research group found a trend toward improvement in kidney quality after prolonged perfusion(24 hours) (39).

Graft Treatment During Machine Perfusion

Hypoxia impairs the endothelial cell function, promotes inflammation and procoagulant activity and leads to vascular convergence, which is considered key to IRI (40–42). Although

these processes can be avoided to some degree with the use of unfractionated heparin, the risk of systemic bleeding must still be considered. Semisynthetic antiplatelet and anticoagulants (APACs) have been found to protect the kidney from moderate and severe IRI by reducing adaptive immune activation and vascular injury, but the systemic risk of systemic administration still needs attention (43, 44). The MP, particularly NMP is more than a preservation benefit to the graft, and more importantly, treatment and handling of the graft before transplantation can be performed during the perfusion period, further improving graft efficacy (45). It may be a more effective way to use mechanical perfusion as a vehicle combined with drugs targeting endothelial cells(ECs) to help reduce the local IR response. Hamaoui et al. used thrombalexin (TLN) for local anticoagulation during HMP, which confirmed the feasibility of HMP as a vehicle. This method promoted the adhesion between TLN and its target to improve organ perfusion (46).

Nanotechnology Combined With Machine Perfusion, 1 + 1 > 2

A rather promising direction for combination nanotherapeutics during machine perfusion. Machine perfusion provides delivery motivation for nanotherapeutics, whereas nanotherapeutics can accomplish treatment of more targeted pretransplant grafts during machine perfusion.

Devalliere et al. used biodegradable PLGA polymer nanoparticles to wrap microRNA-132, which can promote angiogenesis. At the same time, this complex was combined with cyclic RGD (cRGD) peptides to promote its uptake by ECs, and was then implanted into ECs before transplantation, and then the collagen-fibronectin-based scaffold was used to control the transplantation site of ECs. The slowly released RNA then continuously promotes angiogenesis at the target site (47).

Nanoparticles will combine with proteins in organisms to form a coating called protein corona on the surface. This reaction will greatly hinder the normal function of nanoparticles, and may also cause the death of endothelial cells (48). The use of NPs to treat grafts during mechanical perfusion can help prevent the aggregation of NPs in the macrophage system and the "protein corona" caused by serum proteins. Even so, there is still room for improving the targeting ability of NPs. Ensuring that NPs accumulate in target cells to influence perfusion remains challenging. Tietjen et al. bound the anti-human CD31 antibody to NPs and applied it to human kidneys during the machine perfusion phase. The experimental results showed that, compared with non-targeted NPs, targeted NPs had a significantly enhanced enrichment effect, but this effect was weakened by nonspecific aggregation. The nonspecific aggregation of NPs is particularly significant in areas with insufficient blood perfusion, which is a key factor hindering the role of therapeutic NPs. But, however, it can provide direction for predicting the therapeutic effect of NPs or evaluating organ function in the future (49).

Improving the targeting effect of NPs during NMP requires not only modifying NPs but also improving the microenvironmental status of the organ itself. Areas of hypoperfusion were found to have significant microvascular obstruction. Indeed, DiRito et al. found that the microvascular obstruction in the kidney was due to fibrin (ogen) from the proximal tubular epithelium, and treatment with a tPA +plasminogen regimen prior to transplantation resulted in improved microvascular obstruction and vascular targeting capability of the NPs (50). During NMP, a large number of nanoparticles are released, including kidney-derived extracellular vesicles (EVs). These nanoparticles can help clarify the status of the kidney and are a promising means of conducting a evaluating the renal function before transplantation (51).

As an effective means of improving the outcome of transplantation, nanotherapy before transplantation has attracted increasing attention. Zhu et al. used micelles to encapsulate rapamycin and modified the particles to enable targeting of endothelial and epithelial cells. The addition of targeted rapamycin micelles (TRAMs) to standard organ preservation solutions as a pre-transplant preservation regimen helps prevent downstream organ dysfunction and protects cells *in vitro* while preventing the vascular injury and fibrosis resulting from chronic rejection *in vivo* (52). PACE nanoparticles loaded with siRNA targeting CIITA were able to be delivered into organs *via* machine perfusion. Those nanoparticles enabled the efficient encapsulation and controlled release of siRNA, thereby facilitating the prolonged ongoing silencing of proteins of interest to protect organs from acute rejection after transplantation (53).

The importance of graft treatment before transplantation is self-evident, especially for DCD organs. We should thus seek out new renal-related nanoparticles that are more useful for evaluating the renal quality during perfusion, improving the microcirculation according to the evaluation results and then conducting NMP combined with NPs for targeted pre-transplantation treatment in order to further improve the graft prognosis.

NANOTECHNOLOGY IN ARTIFICIAL BIOLOGICAL ISLETS

Islet Transplantation of Patients With Type 1 Diabetes Mellitus (T1DM)

T1DM is a heterogeneous autoimmune disease characterized by a decrease in the number of pancreatic β cells, resulting in insulin secretion dysfunction (54). For such patients, long-term glycemic control, disease management and complication prevention are important. Long-term use of exogenous insulin is an effective way to control blood glucose in such patients; however, while several options are currently available, adverse effects, such as injection site pain, inflammation and hypoglycemia, cannot be avoided (55). In contrast, islet transplantation is believed to be promising for refining physiological insulin secretion and glycemic control, thereby avoiding long-term dependence on exogenous insulin and associated complications; furthermore, it is less demanding than pancreas transplantation, as patients do not need to undergo major surgery (56, 57).

The Edmonton protocol confirmed islet transplantation as a promising approach for restoring endogenous insulin secretion in T1DM to control blood glucose; however, this regimen was unable to maintain insulin independence in the long term and still required the administration of strong immunosuppressive therapy (58). The side effects of long-term immunotherapy cannot be ignored, so how to further reduce the graft's immune response within the recipient is the primary issue to address in order to improve the outcomes of islet transplantation.

Microencapsulation

Encapsulation of islets with semipermeable membranes for immunosuppressive sequestration is a promising approach to achieving immunosuppressant-free transplantation (59). Lim and Sun were the first to use sodium alginate to encapsulate pancreatic islets, and their method prevented the direct contact of immune components with the islet cells without the use of immunosuppressive agents (ISAs), thereby reducing the immune response while not affecting the nutrient uptake by the pancreatic islets (60). Most subsequent studies achieved control over the cyst wall thickness and microcapsule size, improved the overall durability and reduced toxicity of the material itself for the islets of Langerhans by optimizing the encapsulated material (61–63). However, due to biocompatibility issues, while direct immune reactions with cells are avoided, the body's nonspecific foreign body reaction to the microcapsules can lead to the proliferation of fibers, which can lead to disorders in the communication of the islets with external nutrients and eventually cause necrosis of the islets within the microcapsules (64, 65).

Reducing the Immunoinflammatory Response Around Microcapsules

Anti-inflammatory-related drugs have benefits in reducing fibroproliferation, but the extent to which different drugs are effective is unknown, and adverse drug reactions caused by the way in which they are administered systemically are difficult to avoid. To this end, Dang et al. performed an intravital subcutaneous screen of 16 anti-inflammatory drugs and found that dexamethasone and curcumin had the strongest inhibitory effect on early inflammatory proteases and reactive oxygen species (ROS). Therefore, co-encapsulating the drug with the islets in alginate microcapsules, confirming that localized drug release inhibited fibrosis around the microcapsules can improve the control of blood glucose by the microencapsulated islets (64). This issue was similarly explored by Azadi et al., who designed an alginate/dextran-spermine microcapsule encapsulating pancreatic islets along with pentoxifylline (PTX), an anti-inflammatory drug. This therapeutic structure was not only able to effectively shield the islet cells inside the microcapsules but also alleviated the inflammatory response around the microcapsules and reduced fibroproliferation (66).

Nanoparticles in Microencapsulation of Pancreatic Islets

Approaches to reduce the immunoinflammatory response around microcapsules, such as by optimizing the material to improve biocompatibility or by adding anti-inflammatory-related drugs, are reassuring for improving the outcomes of microcystic islet transplantation. Nevertheless, a large number of empty microcapsules may be left behind during islet microencapsulation, which increases the overall graft volume and leads to a stronger host immunoinflammatory response.

Espona et al. combined superparamagnetic iron oxide nanoparticles (SPIONs) with microfluidic technology to purify magnetically labeled pseudoislet-containing microcapsules in order to achieve automated screening of empty microcapsules, thereby reducing the probability of technical errors, and the microcysts that remained after screening greatly reduced the graft size (by over 75%) (67). Large volumes of capsules containing islets still need to be invested in order to achieve therapeutic purposes. The retrieval of these capsules after graft failure or loss of the graft function is an issue that needs to be urgently addressed. Delcassian developed a new encapsulated cell therapy with capsules that could be retrieved magnetically after transplantation by loading carboxylated iron oxide nanoparticles (NP-COOH) into encapsulated islet alginate hydrogels. This strategy preserved the encapsulated functionality (i.e. no need for immunosuppressive therapy for six to eight weeks) while guaranteeing the functionality of the transplanted islets, and no long-term leaching of the nanoparticles was noted (68).

Layer-by-Layer (LBL) Polymer Self-Assembly

Although microencapsulation has proven effective in islet encapsulation, the issue of an excessive volume of microcapsules remains difficult to overcome due to the fabrication process (69, 70). An excessive microcyst volume can affect islet cell metabolic processes and limits the choice of transplant site, such as preventing transplantation to the most desirable site—the portal vein—and forcing the selection of sites such as the peritoneal cavity, where blood supply is not abundant (71), ultimately risking graft failure due to hypoxia. A much more refined encapsulation strategy is therefore needed to solve this problem.

LBL polymer self-assembly enables the preparation of nano-sized thin film coating and is a reasonable approach for downsizing encapsulated islets, and can effectively block the attack of immune components (**Figure 2**). Wilson et al. constructed a nano coating rich in PEG through LBL self-assembly of poly(L-lysine)-g-poly (ethylene glycol) (biotin) (PPB) and streptavidin (SA) and found that this coating did not cause a loss of islets while guaranteeing the immune isolation of islet cells. At the same time, nutrient exchange was not affected, nor was the islet viability or function (72). This encapsulation method can be performed in the nanometer range without concerns regarding diffusion barriers while at the same time greatly reducing the volume of transplanted islets, thereby allowing transplantation to the portal vein.

The feasibility of LBL islets was first validated in an NHP model by Haque et al. They covalently grafted three layers of PEG onto the surface of pancreatic islets *via* LBL technology, achieving a nano-shielding effect. The shielded islets were

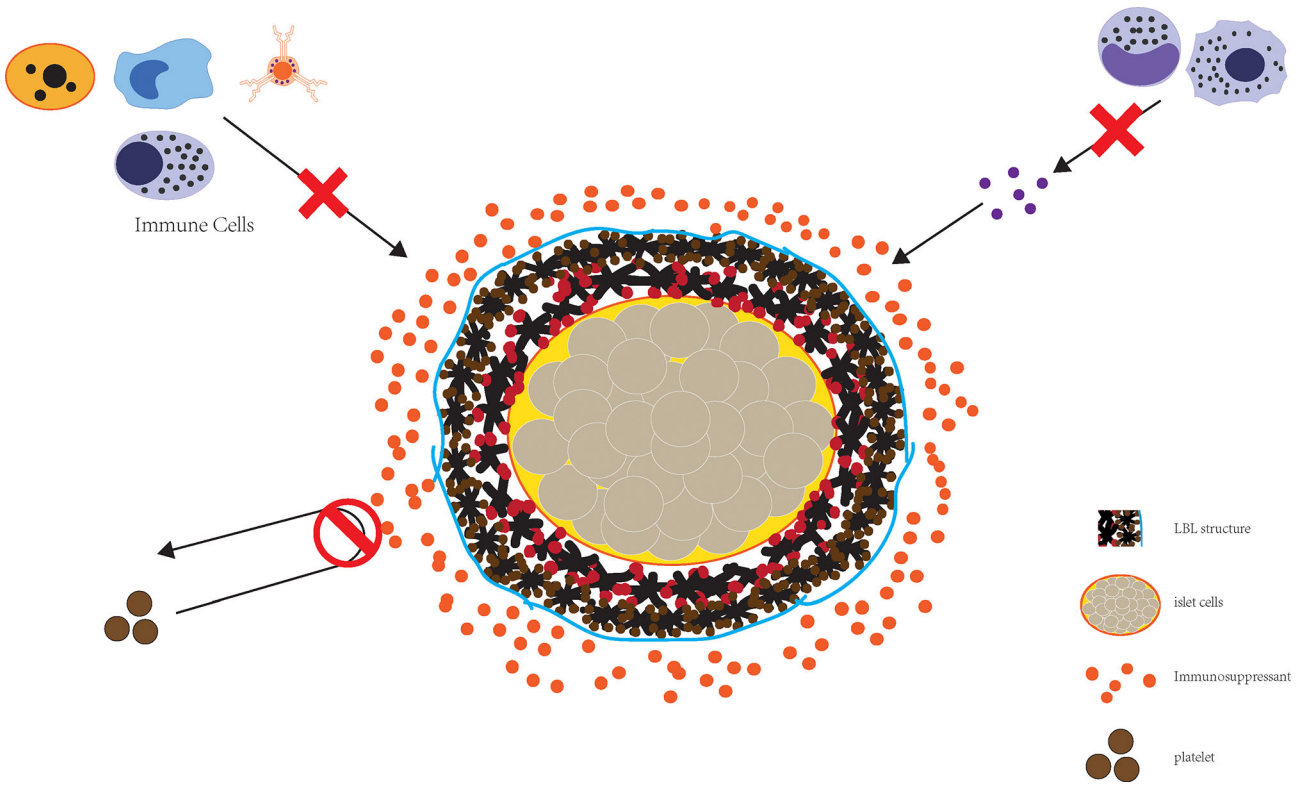

FIGURE 2 | The combined application of LBL structure and immune preparation can effectively realize the destruction of islet cells by immunity.

subsequently transplanted, and studies showed that the LBL islets were less immunogenic, did not have their activity or function affected, had a higher survival rate than non-LBL islets and suffered relatively little immune damage after the application of a glucocorticoid-free immunosuppressive drug regimen, demonstrating the feasibility of this transplantation strategy (73).

To improve the transplanted islet function, Kizilel constructed a nano thin multilayer film of biotin-PEG-NHS/SA/biotin-PEG-GLP-1 *via* the LBL method. This biofunctional multilayer film immobilized GLP-1 on the surface of pancreatic islets and not only improved the islet survival but also promoted insulin secretion, and the response time to glucose was not affected. This coating may reduce the number of islets required, which may help mitigate issues associated with the shortage of islet donors, while also decreasing the volume of the graft and thereby reducing the risk of an elevated portal pressure after transplantation (74).

Reducing Inflammatory Damage

The instant blood-mediated inflammatory reaction (IBMIR) is a major cause of early and massive loss of intraportal transplanted islets and must be addressed in order to successfully initiate intraportal islet transplantation. This problem attracts the attention of a wide range of researchers, and many protocols are proved to be effective in reducing inflammatory injury (**Table 1**). Im et al. used 8-arm-PEG-catechol (PEG8) and N-hydroxysuccinimidyl-linked unfractionated heparin (UFH-NHS) to construct a dual-islet shielding system that effectively inhibited the transplanted islet mediated immune response and IBMIR, an effect that was further strengthened by adding the immunosuppressive agent FK506 (75). Haque et al. used polyethylene glycol plus heparin (heparin nano-shielded islets; HNSIs) in an LBL fashion to shield NHP islets, a protocol that not only blocks islet damage by immune cells but also functions in combination with most current immunosuppressive regimens, demonstrating its reliability in NHP models (76).

Islets remain vulnerable for most of the early period after transplantation due to the loss of their microenvironment. Restoring this microenvironment would thus be conducive to the islet survival and functional recovery. Therefore, Haque et al. constructed a hyperbranched polyethylene glycol (hb-PEG)/Heparin(Hep) nano-encapsule to promote the islet cell survival by shielding them from immune attack, a strategy that reduced IBMIR injury by mitigating complement activation and proinflammatory cytokine generation. This regimen in combination with immunosuppressive drugs had an even stronger effect of preserving the graft survival. The effect of hb-PEG/Hep nano-encapsules on the reestablishment of the islet microenvironment is not a direct effect, instead indirectly helping the islets recreate their own microenvironment by supporting the islet survival (77). Unlike the native

TABLE 1 | The strategies of reducing inflammatory damage.

Author	Years	Method	Type of NPs	Model
Im, Bok-Hyeon et al.	2013	LBL	PEG8+UFH-NHS	Human
Haque et al.	2018	LBL	HNSI	HNP
Haque et al.	2018	LBL	Hb-PEG/Hep	HNP
Izadi Zhila et al.	2018	LBL	JAG1-PEG	Mice
Kozlovskaya et al.	2012	LBL	Tannic acid/N-vinylpyrrolidone	Human/NHP
Abuid et al.	2019	LBL	CONPs/alginate hydrogels	NHP
Syed, Farooq et al.	2018	MLBL	Chitosan/PSS	Human
Gattás-Asfura et al.	2020	CLBL	PAMAM	Mice

environment under physiological conditions, transplanted islets are often in an inflammatory microenvironment, which directly affects the islet transplant survival. Izadi et al. developed a method for modifying the islet surface by coupling NHS groups on PEG to primary amines in the Jagged-1 (JAG-1) structure to immobilize JAG-1 on the surface of islets PEGylated, and these JAG1-PEG islets showed a greater ability to activate the Notch signaling pathway than untreated islets. Increasing CD4+, CD25+ and FOXP3+ cells as well as the IL-10 and TGF-β production while decreasing the INF-γ and TNF-α generation, which reverses the inflammatory microenvironment to an anti-inflammatory one, promotes the formation of an immunotolerant microenvironment that protects pancreatic islets from inflammatory injury (78).

Exploring More Efficient LBL Technologies

Kozlovskaya et al. used a non-ionic hydrogen-bonded LBL technique to produce a novel and versatile cytoprotective coating that is more efficient than the conventional ionic LBL-based method. This method can be performed in a physiological state, and the resulting islets are uniformly coated with a non-toxic and non-immunogenic layer. They can stably exist *in vitro* without affecting the islet function or viability, supporting the modification of functional molecules and also playing a role in inhibiting proinflammatory cytokine synthesis (79).

An insufficient antioxidant capacity of islet cells and oxidative stress damage after transplantation are important causes of a poor function of transplanted islets as well as their death. Abuid et al. generated a nanoscale antioxidant coating through LBL assembly of cerium oxide nanoparticles (CONPs) and alginate hydrogels, a strategy that effectively and durably protected the encapsulated cells from ROS-mediated damage while preserving the cellular metabolism and ability to respond to glucose stimuli. The effect of protection correlates with the number of layers, and it has been shown that 12 layers of CONP/alginate hydrogels coatings have a stronger effect than 2 or 6 layers (80).

Syed et al. used electrostatic bonding technology to deposit two differently charged polymers (positively charged chitosan and negatively charged polystyrene sulfonate sodium salts [PSS]) for the multi-layer-by-layer (MLBL) encapsulation of islets. The MLBL islet activity and function were unaffected, and cells were protected from inflammatory cytokine damage. The transplanted islets showed an improved glucose tolerance and had a significant ability to reduce and stabilize blood glucose levels. A longer time after transplantation can also ensure the survival and morphology of islets and reduce the infiltration of immune cells (81).

The LBL method for constructing pancreatic islet cell coatings often utilizes electrostatic interaction, a promising and helpful approach, but the long-term stability and biocompatibility are issues that remain to be resolved. Gattás-Asfura et al. explored the engineering covalently-stabilized LBL (CLBL) coating approach, which used triethoxysilane with a functionalized poly (amino amides) (PAMAM) dendrimer to enhance the coating stability. Encapulation *via* the CLBL approach allowed for islet engraftment at sites such as the kidney capsule, with no significant difference from untreated islets in the ability to restore normoglycemia or stabilize blood glucose levels and also support islet re revascularization (82).

Nanotechnology in Islet Xenotransplantation

Despite continuous advances in technology for islet encapsulation, the paucity of islet donors remains a barrier to the widespread use of artificial islets. Porcine insulin is structurally very similar to human insulin, and the porcine islet supply is not an issue at present, as access is very easy to achieve, making porcine islets a reliable replacement for human ones (83, 84). Porcine islets are derived from high-quality donors, unlike human donors, who are often elderly or deceased, and porcine islets can be modified by genetic engineering to reduce the immune response, resulting in an improved transplant success (85).

However, immune rejection remains a major obstacle to porcine islet xenotransplantation. Matsumoto et al. used alginate to wrap neonatal porcine islets and implanted them intraperitoneally in patients with T1DM in an experimental group. Their findings suggested undetected porcine endogenous retrovirus (PERV) infection in all subjects, with the HbA1c level maintained at <7%, thus significantly reducing hypoglycemic events. Longer-term maintenance was feasible, as no ISAs were applied, suggesting that immune shielding of porcine islets is an effective approach to address xenogeneic rejection (86). Lew et al. constructed exenatidel-loaded poly (lactic-co-glycolic acid) microspheres after determining the suitable drug-loading capacity and coencapsulated them in alginate microcapsules with porcine islets. The addition of exenatide reduced the rate of islet cell death and improved the stimulatory response to glucose, further enhancing the transplantation success of microencapsulated porcine islets (87).

LBL technology-based nanofilms as well as islet surface modification are an additional option for porcine islet xenotransplantation. Haque et al. combined SH-6-arm-peg-lipid and gelatin-catechol to construct a bilayer structured artificial extracellular matrix for encapsulating and stabilizing porcine islets against cell detachment. In parallel, the use of PEG-derivative encapsulation for islet camouflage reduced the overall immunogenicity and adhesion of serum albumin, fibronectin and immunoglobulin G (IgG). A cocktail immunosuppressant treatment regimen has been adopted after xenoimplantation, which can further inhibit the activation of immunity (83).

Neonatal porcine islet-like cell clusters (NPCCs) are popular because of their low price, ease of isolation and proliferative capacity, but they cause early loss of the graft and subsequent graft failure due to a transplant immune response, such as IBMIR or hyperacute rejection (88, 89). Nanoencapsulation can cope well with these problems, but it is still inescapable for damage in NPCCs encapsulation. Lee et al. focused on how to optimize nanoencapsulation for NPCCs to guarantee the quality of encapsulated islets and avoid unnecessary islet loss. They obtained more stable nanocapsules by inducing cross–linking between bifunctional psomes (NHS-/NH2 psomes) and also found that entrapment in F-10 medium at pH 7.3 could reduce the NPCC damage, while a higher yield of NPCCs could be achieved after adding 0.25% bovine serum albumin (90).

Other Applications of Nanotechnology in Islet Transplantation

Nanotechnology attempts in optimizing islet transplantation are multidirectional and multiangular. Bilirubin is a powerful antioxidant, and its intravenous administration has been shown to reduce apoptosis and levels of inflammatory mediators in order to increase the graft survival in a murine transplant model. However, bilirubin is not readily soluble in water under physiological conditions and has a low bioavailability, and its repeated intravenous injections to maintain effective concentrations carry a risk of toxicity (91–93). Fullagar et al. encapsulated bilirubin into Pluronic F127-chitosan NPs to construct bilirubin nanoparticles. This nanodrug delivery strategy increased the bilirubin uptake and bioavailability by pancreatic islet cells and provided protection to pancreatic islet cells during times of oxidative stress (94). Huang et al. tried to control the insulin secretion process from the implanted islets. They entrapped islets using SA-PEI-melanin composite threadlike hydrogels, an approach that has good biocompatibility and enables effective immune shielding. The introduction of melanin nanoparticles can stimulate insulin secretion and further regulate insulin secretion *via* near-infrared (NIR), achieving a better control of blood glucose levels. Furthermore, they also have retrievable functions (95). Cheng et al. developed an efficient method of islet cryopreservation by combining microfluidic encapsulation systems, cold-responsive nanocapsules (CR-NCS) as well as calcium alginate hydrogel (CAH) embedding. Calcium alginate substitutes traditional toxic CPAs as islet cell cryoprotectants. CAH is responsible for protecting cells during the freeze-thaw process while providing a barrier to the transplanted islets. The islet cell function was preserved under this strategy, and a good glucose-lowering effect was still seen after implantation into diabetic rats. This method is expected to aid in the establishment of islet tissue banks, solve the issue of time lag between donors and recipients and help further mitigate the donor shortage (16).

Furthermore, pancreatic tissue engineering is a promising area for islet transplantation. Elham hoveizi et al. obtained nanofibrous PLA/Cs scaffold by electrospinning technique and incorporated zinc oxide nanoparticles (nZnO) into this 3D culture structure. The results demonstrate that this strategy can provide a favorable nano environment for Endometrial stem cells (EnCSs) differentiation into insulin-producing cells (IPCs) (96). Yung Chih Kuo's group then found that pancreatic islet cells could be generated from IPCs in activin A-grafted gelatin-PLGA NP scaffold by controlling appropriate concentrations of activin A, LY294002, and retinoic acid (RA) (97).

For patients with chronic pancreatitis, on the one hand, long-term chronic pain seriously affects the quality of life of patients. On the other hand, chronic inflammation leads to progressive destruction of pancreatic islets and finally causes diabetes. Total-pancreatectomy (TP) with intraportal-islet-auto-transplantation (IAT) is one of the options for the treatment of chronic pancreatitis. This treatment modality provides pain relief and preserves meaningful islet function (98–100). The introduction of nanotechnology to improve the effect of autologous islet transplantation is a worthy direction for further optimization of this therapeutic approach.

From the use of alginate as a representative microencapsulation tool to the application of LBL technology for successful transplantation from allogeneic to xenografts, from NHP models to humans, from the optimization of encapsulation materials to emphasizing the encapsulation process, optimization of islets themselves, and islet preservation techniques, the continuous advancement and optimization of artificial biological islets have made islet transplantation an important strategy for the treatment of T1DM.

APPLICATION OF NANOPARTICLE CONTRAST AGENT IMAGING FOR ORGAN TRANSPLANTATION

The rejection between donor and recipient of transplanted organs and the functional quality defects of transplanted islets may eventually lead to the failure of the whole transplant treatment program. Therefore, it is necessary to perform timely intervention according to the real-time state of the transplanted islets.

Real-time monitoring of the activity and function of the transplanted islets is critical for the treatment of T1DM. At present, the evaluation of the islet graft function mainly depends on the measurement of clinical biochemical indexes, including C-peptide levels, fasting and stimulated glucose levels and oral/intravenous glucose tolerance testing (101). However,

these indexes can only indirectly reflect the functional status of islet grafts. Therefore, the establishment of a non-invasive real-time quantitative technique for monitoring the islet graft survival is important for the clinical application of islet transplantation (**Table 2**).

Magnetic Resonance Imaging (MRI)

MRI is a reliable non-invasive imaging method for monitoring the efficiency of islet transplantation. It has outstanding clinical benefits, such as no harm to the patient due to ionizing radiation, a high representativeness of reuse, a high imaging resolution, deep tissue penetration and strong tomographic imaging ability. It has been widely used in the imaging-based monitoring of islet transplantation (102).

Superparamagnetic Iron Oxide (SPIO)

Because of the similar density of pancreas and liver on MRI, it is difficult to observe islet cells transplanted through the hepatic vein, so contrast agent is needed to distinguish them. Although islet cell labeling requires an effective uptake of MRI contrast agents, common commercial magnetic nanoparticles cannot be effectively introduced into cells, which has a great impact on imaging.

SPIO has become a widely used MRI contrast agent in clinical settings because of its low toxicity and high sensitivity (103). Koblas et al. showed that the labeling of islet cells with SPIO had no effect on the survival rate, activity or function of transplanted islets. Subsequently, Toso et al. confirmed for the first time in clinical practice that real-time imaging monitoring of islet cells by MRI with SPIO labeling was completely safe and feasible (104). Koichi et al. developed six kinds of magnetic iron oxide nanoparticles coated with different dextrans. The results suggested that these new positively charged nanoparticles might be useful MRI contrast agents for monitoring the islet quality after transplantation, thus confirming that MRI using SPIO nanoparticles contrast agent is a viable tool for *in vivo* monitoring of the islet quality (105).

Some studies on MRI have shown that, after labeling islet cells with these new positively charged nanoparticles, the T1- and T2-weighted imaging signals of islet transplantation sites in mice decreased significantly. However, this result is caused by the transplantation of islets into the subcapsular space of the kidney. If the islets are placed in the porta hepatis commonly used in clinical settings, more artifacts will occur due to the high iron content in the liver (106). This is an urgent problem to be solved in the application of this new positively charged nanoparticle technology in MRI.

In the study of Toso et al., the intensity of the images of three patients before islet transplantation was normal, and the iron-loaded islets were recognizable as low-intensity spots in the liver after transplantation. Another patient had a diffuse low-signal image on baseline liver MRI, so transplant-related changes could not be observed. Iron overload (spontaneous or induced) is the main obstacle to MRI with iron-containing contrast medium (104). Researchers have thus conducted a large number of studies on the modification, improvement and replacement of SPIO contrast media, with some breakthroughs made, and a large number of new SPIO contrast agents have been developed and put into clinical use.

IBMIR is one of the main inflammatory reactions that occurs after pancreatic islet transplantation through the hepatic portal vein, leading to the activation of platelets as well as the coagulation and complement systems. This platelet-amplified reaction creates activated platelets bind to the islets and contribute to a continuous fibrin formation, which can damage the transplanted islets, potentially causing an early function loss, and poses a serious challenge for clinical islet cell replacement therapy. To prevent such islet transplantation failure, a new type of heparin-immobilized HSPIO nanoparticles with anticoagulant activity was developed, and its chemical coupling can be used to camouflage the surface of islet cells. Yong et al. found that HSPIO nanoparticles were able to prevent IBMIR *in vivo* and *in vitro*, and the implantation of such labeled islets significantly reduced the blood glucose level of diabetic animals (107). Such HSPIO was also shown to be quickly eliminated after portal vein injection, did not accumulate in the liver, and was able to be used to track fixed islets, showing both MRI sensitivity and anticoagulant activity. It therefore seems to be a promising clinical choice for a new type of SPIO contrast agent with remarkable future prospects.

Ultra-Miniature SPIO (USPIO)

The biggest difference between USPIO and SPIO lies in their diameters: the diameter of USPIO is less than 50 nm, while that of SPIO is more than 50 nm. As a biodegradable preparation, USPIO is safer than SPIO and non-toxic, showing a long blood half-life, good biocompatibility, and an ability to be swallowed by macrophages without cell activation.

However, most reports thus far on USPIO have focused on the evaluation of kidney transplantation. Stoumpos et al. found that ferumoxytol-enhanced magnetic resonance angiography (FeMRA) was feasible and practical for vascular imaging in patients with advanced renal transplantation. It has the advantage of obtaining arteriography and venography findings at the same time without inducing nephrotoxicity (108). Maryam et al. found that ferumoxytol was not retained in renal allografts in patients with acute rejection, and that renal allografts with acute rejection showed a prolonged T^2 * value compared with non-rejection allografts (109).

It was recently reported for the first time that the USPIO nanoparticle Ferumoxtran-10 can be used in the clinical treatment of islet inflammation in patients with T1DM. In addition, it is a feasible contrast agent for the commercial use of USPIO, receiving approval from the US Food and Drug Administration (FDA) in 2005 (110). Bin et al. found that using a new type of Bcl-2-functionalized PEG-USPIO as a molecular imaging agent had great potential for monitoring islets or other cells *in vivo* (111). Sang-Man et al. found that the labeling effect of USPIO-based MRI was not directly suitable for clinical islet transplantation, so they evaluated the feasibility of ferumoxytol in islet MRI by multi-layer surface modification. They found that multi-layer islet surface modification was a promising choice for portal vein concave islet MRI (112). Therefore, after proper surface modification, the ordinary commercially available

TABLE 2 | Nanoparticles for organ transplant imaging.

Imaging mode	Types	Materials	Characteristics	Application model	Advantage	Disadvantage
MRI	SPIO	/	/	Patients with islet transplantation	/	Iron overload (spontaneous or induced)
MRI	SPIO	ATDM/CMDM/CMEADM/ TMADM-01-05/DEAEDM	The nanoparticles are charged due to the substitution of the cationic terminal group of dextran	SD rats/diabetic nude mice	Effective introduction of cells	The iron content of liver is higher, and more artifacts occur
MRI	HSPIO	/	Anticoagulant activity	SD rats	IBMIR can be inhibited *in vivo* and *in vitro*, which can be quickly eliminated and will not accumulate in the liver	/
FeMRA	USPIO	Ferumoxytol	/	Patients with advanced renal transplantation	Safe and non-toxic, long blood half-life, good biocompatibility, can be swallowed by macrophages without cell activation	High concentrations of reagents may cause artifacts and cannot be directly applied to islets
MRI	USPIO	ferumoxtran-10	/	Patients with type 1 diabetes	Identify the highest risk individuals from occult pancreatitis to dominant diabetes	/
MRI	USPIO	Coated with amphiphilic polymer ([OE-PEG-COOH])	Bcl-2 functionalization of monoclonal antibodies	ICR mice	It can effectively label rodent islet cells and has good distribution and biocompatibility	High contrast medium concentration or inflammatory fluid infusion may cause low signal
MRI	USPIO	Ferumoxytol	A four-layer nanoshield with poly(ethylene) glycol (PEG, 2 layers), ferumoxytol, and heparin was formed on the pancreatic islets	a syngeneic mouse intraportal PIT model and a non-human primate intraportal PIT model	After multilayer modification of islet surface, Ferumoxytol USPIO can be directly used for the labeling of islets	/
MRI	dextran-coated iron oxide MNs	MN-siCaspase-3/MN-siB2M/ MN-miR-216a Probe	Therapeutic/SiRNA nanoparticle probe for dual use	NOD-SCID mice	siRNA gene protection therapy and living body MRI feasibility of non-invasive monitoring of two-in-one combination of transplanted human islets in mice	The trend of particle aggregation hinders their large-scale synthesis
MPI	SPIO	dextran-coated Ferucarbotran SPIOs	/	NOD-SCID mice	An imaging method without depth attenuation and background tissue signal	Lack of background support in physiology and anatomy
19F MRI, CT and US imaging	perfluorocarbon nanoparticles	rhodamine-PFOB/rhodamine-PFPE	Multimode cell contrast agent	mice and rabbits	Combine the advantages of different imaging methods to overcome the limitations of a single imaging method	/
19F MRI, Fluorescence, and Bioluminescence Imaging	PLGA	poly(lactic-co-glycolic acid) (PLGA-NPs) with encapsulated perfluoro-15-crown-5-ether and the near-infrared fluorescent dye indocyanine green	Multimode cell contrast agent	rats	Any false positives in 19FMRI data can be eliminated	Most of them are complex and expensive, and lack a large number of clinical data verification

USPIO should be able to be used to evaluate the status of islet transplant patients as a promising new contrast agent.

Therapeutic Diagnostic Imaging

The term "therapeutic diagnostics" was first proposed by Funkhoer et al. in 2002 (113). Treatment diagnostic imaging is combined with MRI diagnostic imaging and related intervention therapy. It is used to identify specific targets by imaging, design relevant reagents for the targets and visualize them and monitor the treatment response, so as to minimize any effects on normal tissue (114). It can analyze specific information by combining the genome and proteome, enabling the design of a personalized treatment for organ transplantation, which has great prospects and significance for clinical applications in the future.

Wang et al. designed an MR probe by coupling therapeutic siRNA with glucan-coated SPIO and incubated the probe with it before islet transplantation. The results showed that islets transplanted under the renal capsule had a better survival by reducing the expression of caspase-3 in the MN-siCaspase-3 group, while hyperglycemia caused by T cell attack in the MN-siB2M group was significantly delayed (102, 103). Ping et al. synthesized a nano-therapeutic agent composed of magnetic nanoparticles (MNs) coupled with siRNA molecules targeting genes harmful to islet grafts. The combined therapeutic effect provided by an RNA interference technique with an *in vivo* MRI diagnosis is expected to improve the results of islet transplantation significantly in patients with T1DM (115).

Recently, a new kind of nanoparticle was synthesized: MNs targeting phosphatase and tensin homologue (PTEN) coupled to miR-216a. These nanoparticles can down-regulate the expression of PTEN to promote the proliferation of islet β-cells in T1DM animal models (116). However, while these new nanoparticles have the advantage of penetrating the biofilm barrier, particle aggregation trends hinder their large-scale synthesis (117).

Magnetic Particle Imaging (MPI)

MPI is a newly emerging real-time monitoring and imaging method that can directly perform imaging using magnetized iron oxide nanoparticles. It has strong specificity, high sensitivity and safe translation (118). The sensitivity of MPI is 108 times higher than that of the proton magnetization seen in MRI (119). At the same time, MPI is safe and translatable because its carrier iron oxide nanoparticles are biocompatible (120) and do not produce ionizing radiation, and magnetic fields can be used with no threat to patient safety (121).

Ping et al. described the first application of MPI in the monitoring of transplanted islets. SPIO was used to label islets separated from baboons (*Papio hamadryas*). The results showed that MPI was an imaging method without depth attenuation or a background tissue signal, which is suitable for imaging transplanted islet grafts. However, MPI lacks the background support of physiology and anatomy depictions, which is the main issue limiting its clinical application (122).

Multimodal Imaging

The simple use of an imaging mode has obvious limitations, and the observed research results cannot be completely scientifically representative. For example, performing MRI just once results in a low sensitivity, its contrast medium is somewhat toxic, islets must be prelabeled before transplantation, and it is difficult to distinguish between living and dead islets. In contrast, Bioluminescence Imaging (BLI) and Fluorescence Imaging (FI) have disadvantages of poor tissue penetration, a short elimination period, low spatial resolution, propensity to be affected by the internal environment, and a tendency to show weak signals (102). These issues lead to a bottleneck in the clinical promotion of single imaging techniques and fail to objectively reflect the real situation. Therefore, it is very necessary to develop a multi-mode imaging platform that combines the advantages of different imaging methods and overcomes the limitations of individual methods.

Barnett et al. labeled human islet organs with PFOB and PFPE nanoparticles and visualized the islets under the renal capsule of mice and rabbits by MRI, ultrasound and computed tomography (123). The combination of specific and quantitative Fmur19 MRI and sensitive and convenient optical imaging can provide supplementary information to support the distribution and survival of islet grafts. Based on poly (lactic acid-glycolic acid) (PLGA-NPs) wrapped in perfluoro-15-crown-5-ether and NIR fluorescent dye indocyanine green, Gálisová successfully developed a three-mode imaging platform for the *in vivo* examination of islet transplantation (124).However, most of these multi-mode nanoparticles are more complex and expensive than conventional single modality nanoparticles, and the effects of these nanoparticles on transplanted islet cells and human body and their stability need to be explored.

DRUG DELIVERY OF NANOPARTICLES FOR ORGAN TRANSPLANTATION

To improve the success rate of organ cell transplantation, patients need to take ISAs to alleviate the immune response. However, systemic administration of ISAs is associated with serious side effects; for example, the oral and intravenous administration of tacrolimus may lead to systemic toxicity, which greatly reduces the long-term success rate of transplantation (125). The emerging method of nanodelivery may improve the disadvantages of systemic drug delivery *via* the targeted delivery of ISAs to specific organs and tissues.

Improving the Pharmacokinetics and Delivery Efficiency of ISAs

For drugs with a narrow therapeutic index or low bioavailability, intervention with nanoparticle technology can potentially optimize the pharmacokinetics and delivery efficiency. In the early stage of developing transport platforms for ISAS, Edgar et al. found that cyclosporine was needed after renal transplantation, and the pharmacokinetics of cyclosporine were affected by a high lipid value, suggesting the relatively minor influence of a fat-rich meal on the absorption of cyclosporine from Sandimmune Neoral is advantageous (126).. Indeed, Ritschel et al. found that the bioavailability of cyclosporine was

significantly improved when it was used as a microemulsion particle in clinical trials of kidney and liver transplant recipients (127).

At the same time, some studies have found that the release of the mTOR inhibitor rapamycin from poly(ethylene glycol)-b-poly(ϵ-caprolactone) (PEG-PCL) micelles *in vitro* has remarkable benefits (128). A large number of studies have shown that the pharmacokinetic characteristics and delivery efficiency of ISAs can be adjusted by changing the size, shape, surface charge and composition of nanoparticles (129–131). The size of nanoparticles is of great significance in guiding the delivery of drugs to specific transplanted organs (132). After intravenous injection, nanoparticles of different diameters are quickly removed by the kidney, liver and spleen (133–135) or preferentially gathered in the liver and spleen (136, 137). Studies have shown that nanoparticles are injected into the arterial blood supply of organs before transplantation, so the selection of an appropriate particle size will directly affect the intraorgan penetrance (138). Nanoparticles with an appropriate diameter can better meet their target, thereby improving the delivery efficiency of ISAs.

The shape of nanoparticles is also a key factor for determining the half-life of nanoparticles in the circulation. Gentile et al. found that discoidal particles are more likely to marginalize and adhere to the endothelium than spherical particles because of their specific tumbling and edge dynamics (139). Geng et al. found that the circulation time of phyllo-micelles nanoparticles in the body is longer than that of spherical nanoparticles. At the same time, some studies have shown that nanoparticles can affect the internalization of cells due to the change in the phagocytosis rate depending on their external shape, thus improving the pharmacokinetic characteristics and delivery efficiency of ISAs (134).

However, the most important parameter is the surface composition of nanoparticles. Poly(lactic-co-glycolic acid) (PLGA) is a family of biodegradable copolymers, the ultimate degradation products are lactic acid and glycolic acid and easily metabolized by the body. Therefore, it is frequently used clinically as nanoparticles carrying ISAs. ISAs encapsulated within them have the advantages of stability, long circulation time and guided drug release (140). Studies have shown that encapsulating ISAs, such as cyclosporine, tacrolimus and rapamycin, in liposome nanoparticles, such as polyethylene glycol-PLGA (PEG-PLGA), for a controlled release can stabilize the inherent variability of their pharmacokinetics (127, 141–143). High-density lipoproteins (HDLs) can also be used for the surface composition of small, dynamic nanoparticles carrying low doses of statins in order to alter the activity of the immune response through the internalization of macrophages (144). Nanogels coated with intracellular adhesion molecule-1 (ICAM-1) antibodies consisting of a mixture of dextran and lysozyme were found to provide effective drug delivery *in vitro* and *in vivo* (145).

Although we can improve the pharmacokinetics and delivery efficiency of ISAs by artificially setting the above parameters, it is extremely important to ensure the stability of these parameters during drug delivery. Results thus far have shown that the chemical composition of nanoparticles can be maintained using inert metal, graphene oxide or composite surfactant (146–148). As nanoparticles tend to target out-of-organ aggregation after collision, which greatly reduces their delivery efficiency, we need to reduce the aggregation effect in order to improve the stability of nanoparticles (149). These limitations associated with the clinical application are difficult problems that must be resolved in the future.

Targeted Drug Delivery Mode and Mechanism of Nanoparticles

Nanoparticles are often used as movement-targeting agents because they can deliver drugs directly to different locations in tissues and/or cells. Since endogenous autoantigen presentation may have a synergistic effect with targeted inhibition, nanoparticles that deliver ISAs to the target site are a feasible therapeutic strategy. Nanoparticles can target phagocytes (such as dendritic cells) while carrying ISAs or genetic material to achieve therapeutic effects. Alternatively, drug therapy can be achieved by exploring the interaction of ligand-T cell receptor (TCR), which targeting non-phagocytic cells (such as T cells). Nanoparticles can also be used to facilitate the continuous release of autoantigens or to target antigen-presenting cells (APCs) through surface coupling of ligands. In the absence of inflammatory signals, nanoparticles can induce the presentation of self-antigens to APCs, resulting in T cell anergy, failure or tolerance (**Figure 3**). In addition, nanoparticles can transfer antigens and ISAs through surface delivery and/or soluble delivery, resulting in antigen-specific immune regulation (150, 151). Shirali et al. upregulated PD-L1 on dendritic cells *via* the targeted delivery of mycophenolic acid by nanoparticles to prolong the survival time of mouse allografts (152). Shah et al. used an allogeneic skin transplantation model to optimize the transplantation tolerance of the PLG nanoparticle-peptide delivery platform, suggesting that the design of perfect targeted nanoparticles requires suitable immune cell populations (153). Studies have shown that carboxylated 500-nm biodegradable PLG nanoparticles (surface-coupled or encapsulated homologous diabetic peptides) can quickly and effectively restore the tolerance of non-obese diabetic (NOD) mice (154). Nanoparticles have been used to transmit tolerance-causing molecules and cellular antigens, thereby inducing tolerance phenotypes of dendritic cells and promoting the formation of Treg cells *in vivo* (155). Based on the injectable dual-sized microparticle (MP) platform, the immune system can be reeducated in an antigen-specific manner. These particles can be subcutaneously injected into NOD mice to prevent diabetes (156).

In addition, the binding of antigen peptides and red blood cells to antibody fragments *via* nanoparticles promoted antigen processing by scavenging apoptotic cells mediated by bionic red blood cells in a mouse T1DM model (157). Thomas et al. protected islet donors from non-human primates (NHPs; baboons) by silencing a gene responsible for inducing apoptosis (Caspase3), which was connected to a small interference RNA on MNs (158). Targeted antigen transmission can induce immune tolerance, and nanoparticles can be used to improve the effect of

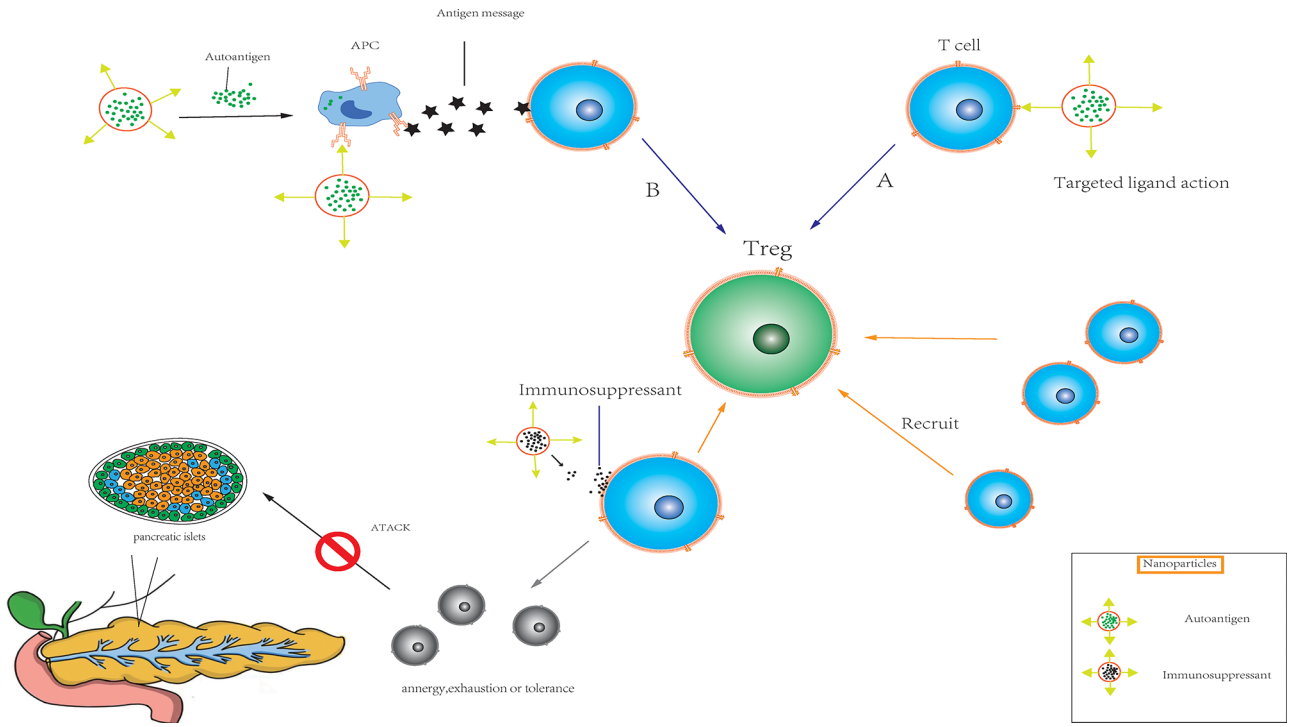

FIGURE 3 | Immunomodulatory nanoparticles **(A)** Nanoparticles target non-phagocytic cells such as T cells in transplanted organs (pancreas, etc.) by exploring ligand-T cell receptor (TCR) interactions. **(B)** Nanoparticles target antigen-presenting cell (APC) through the continuous release of their own antigens or the surface coupling of ligands, and induce antigen presentation to APC. The combination of the two modalities can quickly and effectively activate the target organ to produce regulatory T cells in the absence of inflammatory signals, and recruit a large number of T cells. At the same time, under the combined action of ISAs released by nanoparticles, resulting in T cell anergy, exhaustion or tolerance.

antigen therapy. However, antigen transmission alone may not be sufficient to overcome strong immune rejection and chronic inflammation after replacement with organ cells. The administration of tolerance-inducing drugs in the absence of antigens may lead to non-specific immunosuppression, while the administration of antigens alone may not be effective for treating multi-antigen heterogeneous diseases, such as T1DM. Nanoparticles can be designed to deliver antigens presented on MHC molecules to promote effective antigen presentation and thereby improve specificity and induce inhibition of the T cell phenotype. In the absence of any supportive costimulatory ligands, the treatment of NOD mice with these nanoparticles can inhibit the occurrence of diabetic symptoms *via* the interaction between particles and self-reactive T cells.

CONCLUSION

Continuous advances in transplantation technology are improving the survival of patients with end-stage organ failure, and the addition of nanotechnology is undoubtedly exciting, given the quandary faced by transplantation technology. Whether it be in combination with pretransplant organ preservation *via* machine perfusion, or as artificial biological islets undergoing constant optimization, or through nanoimaging for the *in vivo* survival assessment of transplanted organs, or in the optimization of traditional immunosuppressive drug pharmacokinetics, nanotechnology has been intimately involved in addressing the problems faced by traditional transplantation, and these applications are constantly proving to be extremely promising.

We herein reported advances made in nanotechnology that we feel are inspirational, mainly by discussing the applications of nanotechnology in kidney transplantation as well as pancreatic islet transplantation. However, at present, the application of these findings in the clinical setting is not yet feasible, and the long-term efficacy and safety of nanotechnology need further observation and discussion. The development of nanomaterials and continuous advances in the strategies used for transplantation will ultimately lead to a more favorable prognosis for transplant patients.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing and editing of the manuscript, contributed to the article, and approved the submitted version.

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Islet Encapsulation: New Developments for the Treatment of Type 1 Diabetes

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Islet transplantation is a promising approach for the treatment of type 1 diabetes (T1D). Currently, clinical islet transplantation is limited by allo - and autoimmunity that may cause partial or complete loss of islet function within a short period of time, and long-term immunosuppression is required to prevent rejection. Encapsulation into semipermeable biomaterials provides a strategy that allows nutrients, oxygen and secreted hormones to diffuse through the membrane while blocking immune cells and the like out of the capsule, allowing long-term graft survival and avoiding long-term use of immunosuppression. In recent years, a variety of engineering strategies have been developed to improve the composition and properties of encapsulation materials and to explore the clinical practicality of islet cell transplantation from different sources. In particular, the encapsulation of porcine islet and the co-encapsulation of islet cells with other by-standing cells or active ingredients for promoting long-term functionality, attracted significant research efforts. Hydrogels have been widely used for cell encapsulation as well as other therapeutic applications including tissue engineering, cell carriers or drug delivery. Here, we review the current status of various hydrogel biomaterials, natural and synthetic, with particular focus on islet transplantation applications. Natural hydrophilic polymers include polysaccharides (starch, cellulose, alginic acid, hyaluronic acid, chitosan) and peptides (collagen, poly-L-lysine, poly-L-glutamic acid). Synthetic hydrophilic polymers include alcohol, acrylic acid and their derivatives [poly (acrylic acid), poly (methacrylic acid), poly(acrylamide)]. By understanding the advantages and disadvantages of materials from different sources and types, appropriate materials and encapsuling methods can be designed and selected as needed to improve the efficacy and duration of islet. Islet capsule transplantation is emerging as a promising future treatment for T1D.

Keywords: islet, material, immunogenicity, encapsulation, transplantation

INTRODUCTION

Diabetes mellitus (DM) describes a group of metabolic disorders characterized by high blood glucose levels, and patients with DM have an increased risk of developing a number of serious life-threatening pathologies like retinopathy and cardiovascular diseases (1), resulting in higher medical care costs, reduced quality of life and increased mortality (2). It was estimated that in 2017, there were 451 million (age 18–99 years) people with diabetes worldwide. These figures were expected to increase to 693 million by 2045 (3). Patients with type 1 diabetes (T1D) depend on exogenous insulin supply, however long-term clinical insights have shown the failure of insulin preparations to fully replicate biological actions of endogenous insulin (4). For patients suffering from severe and repeated hypoglycemia events, islet transplantation demonstrated beneficial effects.

Unfortunately, islet allo or xenotransplantation is limited by inflammatory and immune reactions resulting in low survival (5, 6). Transplanted islets are initially destroyed by instant blood-mediated inflammatory reaction (IBMIR) during intraportal infusion of allogeneic or xenogeneic islets (7). Simultaneously, cell transplantation by intraportal infusion may cause bleeding, thrombosis and other related complications (8). The coagulopathy may be due to the release of tissue factor (TF), a physiological trigger for clotting that is secreted by islet cells (9).

Shortly after transplantation, acute immune responses are initiated as chemokines and chemokine receptors induce the recruitment and activation of various leukocytes (10). Activated immune cells cause damage to the islets by releasing pro-inflammatory cytokines and reactive oxygen species (ROS) (11).

Currently, prolonged graft survival is achieved by using continuous immunosuppressive drugs. However, the toxicity associated with long-term immunosuppression includes various adverse effects, such as opportunistic infections, nephrotoxicity, myelosuppression and cancer (12). Immunosuppressive medication is also detrimental to the survival and functionality of the transplanted cells. These severe side effects may significantly reduce the benefit of islet transplantation. Moreover, long-term use of immunosuppressive agents can also significantly impair glucose tolerance and lead to increased insulin resistance (13).

To prevent immune rejection and avoid continuous immunosuppression, cell encapsulation technology is a promising alternative relying on the immobilization of endocrine cells into semi-permeable hydrogel matrices which protect them from the immune system (**Figure 1**). Currently, there are several islet encapsule approaches. The main strategy consists in the use of polymeric hydrogel microcapsules which protect islets from contact and attack by immune cells. Their specific pore size makes the biomaterial membrane permeable to small nutrients and secreted insulin, but prevents the diffusion of immune cells and large molecules such immunoglobulin into the capsule core (**Figure 2**) (14). A complementary strategy is to co-embed islets with by standing cells, molecular cargos and biomolecules that promote islet survival and function. Nevertheless, islet immobilization into hydrogel microcapsules result in the exacerbation of the hypoxic situation (**Figure 3**) (15). Such detrimental effect can be addressed

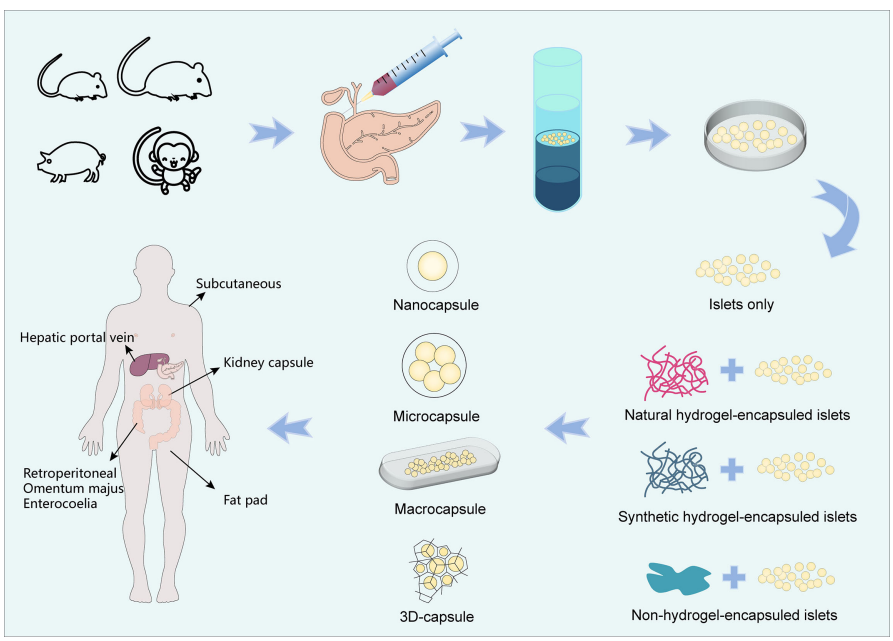

FIGURE 1 | Flow chart of islet encapsulation and transplantation. There are many sources of islet cells, such as mice, rats, porcines and monkeys, etc. We injected collagenase into pancreas and isolated islets by density gradient centrifugation after pancreatic filling. The islets are capsuled with natural hydrogels, synthetic hydrogels and other types of hydrogels, forming capsules of different sizes (Nanocapsule, Microcapsule, Macrocapsule and 3D-capsule) and then transplanted. Common sites of transplantation are subcutaneous, kidney capsule, hepatic portal vein, retroperitoneal, omentum majus, enterocoelia, fat pad, etc.

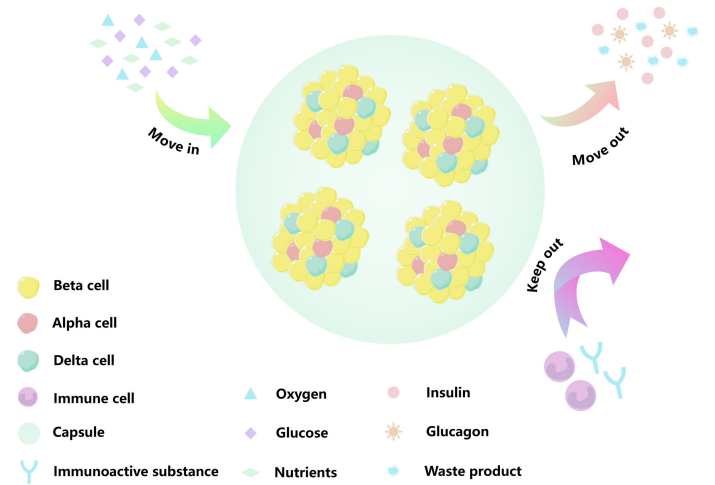

FIGURE 2 | Schematic diagram of an encapsulated islet. Biomaterials can provide shelter for islet cells, minimize the immune response, and mimic a process by which material moves in and out of cell. The yellow cells are beta cells, the red cells are alpha cells, the green cells are Delta cells, the purple cells are immune cells, the green circular background is capsule, the blue Y-shape is immune active substance. The blue triangle is oxygen, the purple square is glucose, the green diamond is nutrient, the pink circle is insulin, the orange sun is glucagon, and the blue star is metabolic waste.

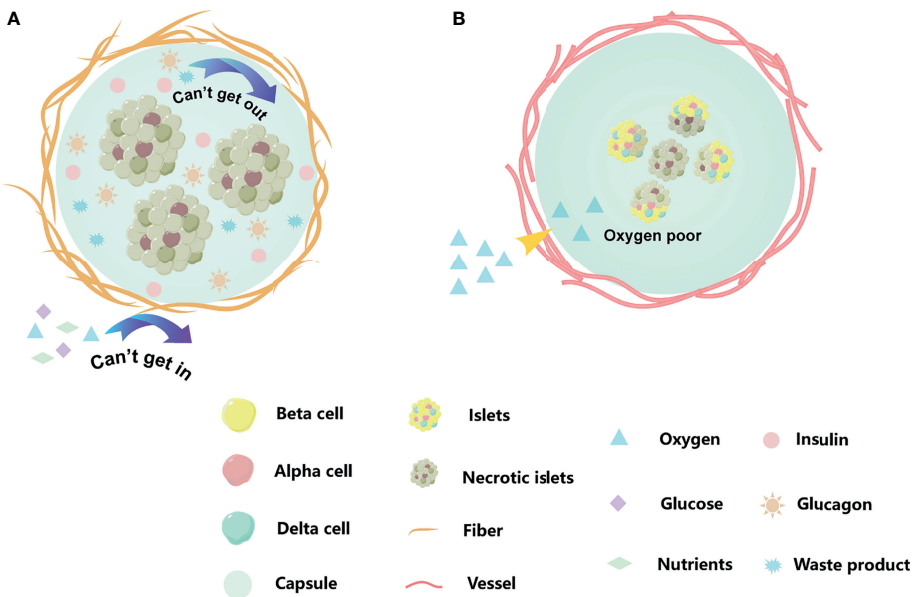

FIGURE 3 | Existed problems for islets encapsulation. **(A)** A dense peri-capsular fibrotic overgrowth, FBR usually limits the diffusion of nutrients and oxygen to the implant, or prevents insulin release and waste discharge. **(B)** The encapsulation we moved into recipient acted as a physical barrier, blocking blood vessels from growing into the capsule. The cell of capsule center by hypoxia. The orange strips are fiber, and the red cords are blood vessel. When islet cells are affected by hypoxia or cellular metabolites, they change from bright yellow to dark gray.

by the use of macrocapsule devices which promote angiogenesis and allow for extracorporeal oxygen supply. Although some researchers summarized the islet encapsulation, they were focusing on different aspects, such as the size of the encapsulated material, immune fusion (16, 17), the immune mechanisms of islet transplantation (18), clinical trials and their influencing factors (19) or the encapsulation of stem cell-derived islets (sc-islets) (20). This review focused on the classification of the islet encapsulation materials, highlighting their application in islet transplantation. In order to better understand the materials, we summarized the designing of the encapsulation materials, the generation of the fibers, and also the related *in vivo* studies.

DESIGN RULES FOR ISLETS ENCAPSULATION

For designing islet encapsulation strategies, a series of parameters needs to be carefully assessed, including the content of the graft, the expected capsule size, the targeted mode of surgical operation, the implantation site, donor-recipient differences, environmental conditions within the organism, etc. The selection and development of the appropriate hydrogel is key to the success of the transplantation. We discuss the various aspects that determine optimal hydrogels selection and design in conjunction with the structure-property-function of stimulating-type polymers (21).

Biocompatibility of Encapsulated Materials

T1D is a complex immune disease. Therefore, biocompatibility is the most important feature in the selection of islet encapsulation materials in order to avoid any deleterious immune response from the recipient. Biomaterial properties such as topography, surface charge and mechanical stability can also be modified to modulate the biocompatibility of the encapsulation matrix toward both the islet content and the host, thus influencing cell survival and recipient tolerance to the implanted material.

Topographical features (e.g., geometric arrangement) may play a role in guiding cell behavior (e.g., adhesion, migration). It has been found that the pore size of the encapsulated material affecting the elongation and transition of macrophages (M1 to M2 phenotype) (22), achieving functional regeneration of tissues (23). Porous matrix can be prepared by various methods such as fiber bonding, salt impregnation, foaming, three dimensional (3D) printing, and freeze-drying (24).

Charge is the determinant of the aqueous environment inside the hydrogel (25). By controlling the hydrogel internal water structure, the solute diffusion rate in the hydrogel can be changed. When the hydrogel has a neutral charged structure, it strongly binds water molecules and effectively inhibits the adhesion of bioactive substances (26). Chemical modification of zwitterionic polymers or charge neutralization of hydrogels with PEI and melanin can achieve better immune-shielding performance (27).

The mechanical stability of hydrogels affects the ability of encapsulated cells to maintain long-term stability *in vivo*. In this case, the mechanical properties of the hydrogel can be regulated by covalent cross-linking, grafting or mixing with other suitable polymer chains to prevent instability under physiological conditions. On the other hand, the stiffness of the hydrogel affects the strength of the foreign body reaction (FBR) it induces *in vivo* (28). It still remains a challenge to produce hydrogels which reach a balance between adequate cell protection to maintain their long-term functionality while causing as little FBR as possible through proper regulation.

pH (*Pondus hydrogenii*)

The pH value of human body fluid ranges from 7.35 to 7.45. A decrease of the pH value by 0.1 results in a decrease of insulin activity by 30%. Therefore, the pH value should be carefully taken into account when designing encapsulation hydrogels. pH affects the properties of hydrogel materials, usually sensitive hydrogels containing anion and cation groups such as -COO-, -NH3+, -OPO3+ accept or give ions through pH changes to achieve gel-solution changes. These changes are mainly attributed to electrostatic repulsion and osmotic forces within the backbone chain (29). The same materials were prepared in solutions with different pH values, and the change in pH value led to a significant change in the interface-cell interactions (30).

Enzyme

In recent years, bioactive hydrogels have been generated by non-covalent co-assembly with enzymes. The different structures and functions of hydrogels are achieved through *in situ* enzyme dynamic cross-linking, enzymatic polymerization and interfacial enzyme assembly effects to build an efficient interoperative responsive microenvironment (31). By scavenging excess enzymes as catalase (CAT) and superoxide dismutase (SOD) in the hydrogel, the islets could have prolonged viability by eliminating reactive oxygen species (ROS) (32). Additionally, there are also metal-containing nano-enzymes, which mimic natural enzyme-like activities with smaller size, enhanced stability and lower price,. However, it is important to note that enzymes, may cause damage to the islet microenvironment and ultimately lead to a poor prognosis even at the initial stage of transplantation.

Temperature

Current islet transplantation still relies mainly on isolation and transplantation from deceased donors. In case of mismatch between donor and recipient time, there is a need to find efficient cryopreservation techniques. Combining cold-sensitive hydrogels with appropriate encapsulation techniques allows islets to not only survive the freeze-thaw process, but also provide a natural barrier to islets *in vivo (*33). In a specific temperature range, thermo-responsive polymers undergo phase transition due to the formation of intermolecular hydrogen bonds, hydrophobic interactions and physical entanglement of polymer chains (34). Conjugation a hyaluronic acid with different sulfation degrees and an amine-terminated poly(N-isopropylacrylamide) resulted in a thermogel that was not only highly compatible with rabbit corneal cells, but its degree of sulfation also had a lasting anti-inflammatory effect (35).

Hydrogel Preparation Methods

Large-Scale manufacturing includes preparing injectable and pre-synthesized hydrogels. Injectable hydrogels enable islet transplantation by injection at the implantation site due to their fluidic nature. The advantages of injectable hydrogels over pre-synthesized solid hydrogels are their non-invasive nature and adaptability to the host tissue space (36). Most injectable hydrogels do not maintain the structural integrity required to protect cells after injection. There can also be gelation during the injection, clogging the pillow and preventing the transplant process (37). When designing injectable hydrogels, attention should be paid to several aspects (36). The first is the shear thinning, which cushions the damage to the fragile cells from shear forces

during injection. The second is thixotropy, which determines the rate at which the injectable hydrogel transforms into a robust protective capsule. The means of triggering also has to be considered, which will determine the suitability of the hydrogel. Injectable hydrogel can be prepared by non-toxic chemical cross-linkers, enzymatic cross-linkers, physical interactions, etc. Alginate has natural, biocompatible and economical properties. However, its potential as an injectable hydrogel is limited by poor control of gelation. Alginate based hydrogels were prepared by ionic cross-linking methods. The physicochemical properties of injectable alginate such as gel formation time, hardness and porosity can be adjusted using different concentrations of Na_2HPO_4 (38). In addition to different concentrations of ions, variation on the ion sources can also modulate the alginate gelation process, resulting in a different range of physicochemical properties. The human body environment and circulating biomolecules were also exploited for the development of injectable hydrogels. The injectable hydrogel obtained by mixing plasma with the hydroxypropyl methylcellulose (HPMC) serves as the base environment for islet encapsulation. When this liquid is injected into the body, it rapidly polymerizes into a fibrin gel after being influenced by circulating thrombin *in vivo*. The nest-like structure formed by the polymerization provides protection to the islets and improves their survival rate (39).

Solid hydrogels, also known as pre-synthesized hydrogels, pre-immerse islets *in vitro*, and the incorporation of extracellular matrix (ECM) components greatly enhance islet functions. Solid hydrogels provide good encapsulation conditions for islet cells to be implanted, but may be accompanied by various types of surgical risks during the transplantation process. At the same time, the already fixed shape is a considerable challenge in adapting to the host's tissue space and mechanical properties, which are necessary to establish host-implantation interactions (40). Prefabricated hydrogels can be prepared from a wide variety of materials, involving various engineering methods and potential combination with biological factors and active substances. These aspects are not detailed in the present review.

HYDROGELS AS BIOMATERIALS FOR CELL ENCAPSULATION

Hydrogels are composed of cross-linked macromolecules that form 3D structures with high water content. Cross-linking strategies include physical interactions (hydrogen bonding), ionic interactions and chemical conjugation which ensure the stability and physical integrity of hydrogels in aqueous medium (41). In addition, the cross-linked network provides the hydrogels with tunable mechanical properties (strength and elasticity) and determines their diffusion properties and internal transport capacity as encapsulation material (42). The polymer network maintains the shape and volume of hydrogels by balancing capillary, osmotic and hydration forces to protect cells (43). At the same time, their high-water content mimics the softness of natural tissues while allowing the bi-directional diffusion of nutrients, metabolites and wastes (44, 45). These characteristics make hydrogel ideal cell encapsulation materials. Most water-soluble or hydrophilic polymers can become hydrogels upon chemical or physical cross-linking conditions. However, the morphology and properties of the final capsules highly depend on the composition of the polymeric materials and the technology applied for capsule formation. A large variety of natural and synthetic materials were investigated in the context of cell encapsulation (**Table 1**).

Hydrogels derive from natural or synthetic polymeric materials (61). Natural hydrophilic polymers include polysaccharides (starch, cellulose, alginic acid, hyaluronic acid, chitosan, etc.) and peptides (collagen, poly-L-lysine, poly-L-glutamic acid), etc.). Synthetic hydrophilic polymers include poly (vinyl alcohol), acrylic acid and their derivatives (poly (acrylic acid), poly (methacrylic acid), poly(acrylamide), etc.). In recent decades, hydrogels have been widely used in wound dressings (62), tissue engineering (63), cell carriers (64), drug delivery (65), antifouling coatings (66).

HYDROGEL DERIVED FROM NATURAL POLYMERS

Hydrogels produced from natural materials mostly derive from polysaccharides such as starch, cellulose, alginate and collagen, as well as peptides such as elastin and poly-L-lysine (67).

Alginate is a polysaccharide mainly found in the cell wall and intercellular mucilage of brown algae, but also in some bacteria of *Azotobacter* sp. and *Pseudomonas* sp. It is commonly applied as cell microencapsulation material due to its low toxicity, low immune response, and ability for instantaneous formation of ionic hydrogel in the presence of divalent cations such as Ca^{2+} and Ba^{2+} (68, 69). Alginate is a linear copolymer composed of 1-4-linked β-D-mannuronate (M) and (or) α-L-guluronate (G) residues. The physical properties of alginate molecules are determined by the ratio and distribution of the three types of blocks: MG-blocks, MM-blocks, GG-blocks (70). The fast gelation properties of alginate upon contact with a solution containing divalent cations allow for cell microencapsulation under mild conditions (68). Several parameters, such as the association constant, the ionic strength and the affinity for the different blocks, depends on the nature of the gelling cations. For instance, Ba^{2+} preferably associates with GG and MM blocks while Ca^{2+} favors ionic interactions with GG and MG blocks. These different association patterns have significant impact on the porosity, rigidity, elasticity and mechanical resistance of the hydrogel (71). The induction of immune responses in organisms by alginate is mainly related to its composition. Several studies highlighted that M-blocks and MG-blocks, but not the G-blocks, stimulated the cytokine production (72). There are claims that immunogenicity is related to the purity of the alginate, and that mannuronic acid-rich alginate is usually less viscous, allowing the gel to have a higher alginate content. Implantation of these purified alginate capsules into mouse models with elevated macrophage activity also showed no FBR (73).

Collagen is a fibrous protein and displays at least one triple-helical domain. It is an important component of the animal ECM and ensures the structural integrity of tissues and organs (74). The mechanical properties and stability of collagen are lower than the natural state due to the disruption of the assembly

TABLE 1 | Hydrogels as biomaterials for cell encapsulation.

Material	Honor	Recipient	Graft	Site	Result	Reference
Alginate	Wistar rats	Wistar rats	2,000~3,000 islets	intraperitoneal	normoglycemia (3 wks)	(14)
Collagen	C57BL/6J or CD1 mice	NOD.CB17-Prkdc^{scid}/J or C57BL/6J	500 islets	subcutaneous	normoglycemia for 14d (immunocompromised), 90d (syngeneic), and 40d (allogeneic).	(46)
Silk	BALB/c or C57Bl/6		islets, ECM and MSCs		islets remained viable and SI was 4.4(7d)	(47)
Alginate&gelatin	double heterozygous crossbreed mice	NSG mice	500 islets	subcutaneous	detect GFP expression and image islets(7d)	(85)
Alginate-poly-L-lysine	Rats	Mice	1000 islets and 1mg MSC-CellSaic	the skin and muscles of the abdomen	a large number of vessels normoglycemia (by 1 month)	(49)
Alginate-polylysine-alginate	Porcine	Monkeys	3-7 x 10^4 islets at a time	intraperitoneal	normoglycemia (120d~804d)	(50)
Alginate&Teflon	German landrace sows	Gottingen Minipigs	3000 IEQ	subcutaneous	no signs of local inflammatory or fibrotic reactions(13d), the Glucose stimulated insulin release (GSIR) is 6.7	(51)
PEG-RGD	Balb/c	B6	1,200 IEQ	Epididymal fat pad (EFP)	normoglycemia (>100d)	(52)
PEG-RGD	B6(GFP)	NOD mice	800 IEQ	gonadal adipose tissue	high density of GFP signal and significant vascularization (9 wks)	(52)
PEG-MAL-RGD	Rat	Rat	1,500 islets	mesentery	new vessels (by 1 wk)	(53)
Biotin-PEGNHS/SA/ biotin-PEG-GLP-1	Rat	_	islets	_	increased insulin secretion	(54)
PEG-b-PLA	Piglets	C57/BL6	neonatal porcine islet-like cell clusters (NPCCs)	kidney capsule	little infiltration of immune cells	(55)
PEG-DA	Porcine	Athymic mouse	islets	kidney capsule	no fibrosis (>2 wks) viable porcine islet(>100d)	(56)
Triazole-zwitterionic (TR-ZW)	Sprague-Dawley rats	C57BL/6	500 islets	subcutaneous	normoglycemia (1 month)	(57)
PEG-inhibitory peptide	_	_	Mouse insulinoma (MIN6) cells	_	reduce the death of MIN6 cells	(58)
PLG	BALB/c	C57BL/6	1,000-1,100 islets	epididymal fat pad (EFP)	normoglycemia (200d)	(59)
PLG-Treg	NSG mice	NOD mice	300 islets+3x10^6Treg	intra-abdominal fat	normoglycemia (>99d)	(60)

structure and natural cross-linking during the extraction process, which requires the induction of exogenous cross-linking for its optimization (75). Cross-linking may lead to collagen denaturation and exposure of antigenic determinants clusters to induce immune responses (75). Moreover, due to the differences in species, natural collagen may cause immune problems such as allergies as well as disease propagation.

Unlike previous collagen formulations used for islet encapsulation, Clarissa et al. (46) used oligomeric collagen to encapsulate islets, which retains natural intermolecular cross-linking and is able to rapidly self-assemble into highly interconnected D-band fibrous scaffolds upon neutralization (76). These structures were applied to the encapsuling of mouse islets. The highest functionality of the embedded cells, measured *in vitro* over 14 days, was achieved at a concentration of oligomer of 3 mg/mL. The ability of these systems to maintain normoglycemia after subcutaneous injection in the back was demonstrated *in vivo* with different models: 14 days in NOD.CB17- Prkdc^{scid}/J mice, 90 days in syngeneic mice and 40 days in allogeneic mice. Islet microorganisms interact with extracellular matrix components such as type I collagen fibers to maintain islet function and homeostasis. The high biocompatibility of type I collagen *in vivo* supports small molecule transport while reducing infiltration and activation of inflammatory cells, providing a new solution for subcutaneous islet transplantation.

Silk comes from a wide range of sources, such as silkworm, spider silk, and peanut silk, which results in differences in the composition of silk protein. Treated silk proteins have low antigenicity and rarely cause immune reactions when implanted *in vivo* (77). The main factors causing an immune response are the immune cells and signaling molecules adsorbed on the material (78). The performance of islets encapsulated in silk materials was significantly enhanced by co-encapsulation with fibroin, a protein presenting strong mechanical properties and low immunogenicity (79, 80). Davis N et al. (47) demonstrated that the co-encapsulation with MSCs resulted in a 2.3 fold increase of the stimulation index (SI) and that additional co-encapsulation of fibroin led to 4.4 fold SI enhancement, as compared with pure silk encapsulated islets.

COMBINATION OF POLYSACCHARIDE AND POLYPEPTIDE POLYMERS

The 3D-printed capsule technology uses polymers as inks and piezoelectric or thermally driven mechanisms to process high-resolution bio-ink droplets (**Figure 4**) (81). The precise spatial control achieved with 3D-printing allows for the immobilization of cells into well-defined microstructures such as cylindrical filaments (82). The variety of complex structures produced through this technology has the potential to overcome the technical difficulties faced by conventional islet encapsulation. In particular, conventional polymeric hydrogel microcapsules generally do not support the formation of a vascular network after transplantation, thus impairing the efficient delivery of oxygen and nutrients to the entrapped cells (83). A few studies investigated the ability of 3D printed scaffolds to promote the formation of a vascular network around encapsulated islets (84).

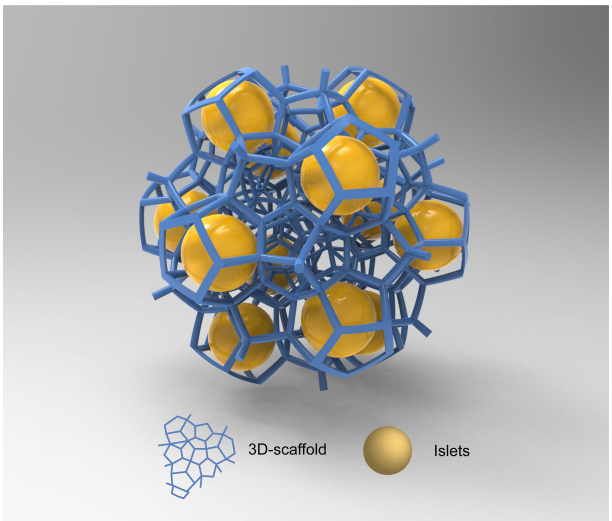

FIGURE 4 | 3D-capsule schematic diagram. Special biomaterials are used as inks to print 3d scaffolds in which cells are housed. The yellow spheres in the figure are islet cells, and the blue scaffold is 3D scaffold.

Marchioli et al. produced 3D-printed hydrogels composed of 4% alginate and 5% gelatin (85). By controlling the shape and porosity of the scaffolds, they observed the formation of blood vessels toward the device and promotion of angiogenesis. Compared with conventional spherical hydrogels, 3D-printed scaffolds prevented islets from aggregation, thereby increasing the surface area for small molecule exchange.

Currently, 3D bio-printing technology is still at an early stage of development, and its application is still subject to many limitations. The relatively weak mechanical properties of bio-inks used in 3D printing does not allow for the formation of hydrogels requiring high mechanical strength (86). The bio-inks used in 3D printing should present low viscosity (<10 mPa/s) to avoid nozzle blockage, thus limiting its applicability (87, 88). Further research is needed to develop hydrogels with sufficient viscosity and mechanical properties to suit the plotting function and islets functionality. At the same time, the incorporation of ECM components, endothelial cells and vascular endothelial growth factor (VEGF) into the bio-ink can make the printed model more similar to the living environment of islet cells (89), thus enhancing their biological function. Otherwise, 3D printing technology can achieve fast manufacturing throughput and maintain high cell vitality. Overall, 3D printing is seen as one of the most promising encapsulation approaches because it can produce clinically relevant multi-component devices in a short period of time.

CO-ENCAPSULATION STRATEGIES BASED ON NATURAL POLYMER DERIVED HYDROGELS

Mesenchymal Stroma Cells (MSCs) reduce the immune response by releasing cytokines and growth factors (90, 91) and also have

the potential to induce angiogenesis and repair of damaged tissues (92). MSC-CellSaic is a cell transplantation platform consisting of MSCs and recombinant peptides (RCP) arranged in a mosaic shape to avoid cell death (93). Ryo Kogawa et al. used CellSaic technology in a two-stage implantation protocol. Fist, an empty mesh bag was placed into the abdominal cavity of Balb/C mice, which could induce the formation of blood vessels around and in the band. Subsequently, alginate microencapsuled rat islets coated with poly-l-lysine (PLL) and MSC-CellSaic were inserted into the nylon mesh bag. Glucose levels were significantly reduced in the mice treated with the combination of islets and MSC-CellSaic as compared with the control group without MSCs (49).

Using alginate-poly-L-lysine-alginate microcapsules, porcine islets were transplanted into diabetic Cynomolgus monkeys. Seven of the nine monkeys achieved normal fasting blood glucose with insulin independence for periods ranging from 120 days to 804 days (50). As mentioned earlier in relation to alginate immunogenicity, mannuronic acid residues act as cytokine inducers causing fibrosis in implanted microcapsules. When alginate microcapsules are covered with PLL, the mechanical stability and permeability of the encapsulation material is improved.

Considering the severe hypoxia and immediate blood-mediated inflammation that portal vein and intraperitoneal transplants face, extravascular islet transplantation has been explored. A device, termed 'β Air' device, consists of islets immobilized in alginate and a hydrophilic Teflon membrane impregnated with alginate. The alginate and membrane barrier protect the islets from contact with immune cells, complements and antibodies, while a subcutaneous port supplies oxygen from the outside. Under these conditions, allogeneic pig islets were explanted after 13 days and the islet function remained active. The transplanted pocket was clean and showed no signs of local inflammation or fibrotic reaction, providing a new strategy for the feasibility of pig islet cell transplantation (51). After testing the device in small and large animal models, the method was applied to a patient with long-term T1D. The chamber with allogeneic islet was placed in a preperitoneal pocket and the oxygen supplying device was implanted close to the incision. The allogeneic islets survived and were able to maintain insulin secretion in response to the variation of blood glucose level for 10 months. A thin fibrous capsule appeared at the implantation site without any signs of inflammation (94).

ARTIFICIAL HYDROGELS

While hydrogels deriving from natural polymers display adequate biocompatibility and low production cost, their stability under physiological conditions is often limited. In contrast, the production of artificial hydrogels, also called synthetic hydrogels, can provide improved control over the material properties, including pore size, mechanical strength and elasticity. Synthetic hydrogels have higher mechanical resistance, extended durability and wider application range as compared with natural hydrogels (41).

Poly (ethylene glycol) (PEG) hydrogels have been widely used in implantable devices due to their advantages such as convenient and rapid preparation. However, PEG is less biocompatible than natural polysaccharides and does not fully protect encapsulated cells from cytokines attacks (95). The combination of PEG with controlled amounts of components of the native islet microenvironment led to promising cell transplantation devices (96). The proteins naturally associated with the islet basement membrane (fibronectin, laminin, type IV collagen) were co-encapsulated with mice islets and resulted in enhanced *in vitro* insulin secretion up to 7 days post-encapsulation (97–99). The effect was further reinforced by the incorporation of bone marrow derived MSCs within the hydrogel matrix (48).

Weber et al. combined murine islets with type IV collagen and laminin in three-dimensional PEG hydrogels resulting in a two-fold and four-fold increase in insulin secretion, respectively, compared with islets that were not encapsulated with ECM proteins. Hydrogel containing both matrix proteins and more than 75% laminin produced about six times higher insulin secretion when compared with the islets encapsulated in the absence of matrix proteins (98).

Arginine-glycine-aspartic acid (RGD) is present in several ECM proteins, such as fibronectin, collagen type I, allowing for cell adhesion and its modified substance improves biocompatibility (100). Recent studies have shown that the incorporation of oligo-peptide RGD into PEG hydrogels reduces FBR (101). Weaver et al. have developed a microfluidic system, that produces smaller diameter microgels (310 + 14 μm) based on synthetic PEG, allowing their implantation within a vascularized retrievable site. Under these conditions, murine islets were encapsulated in PEG-RGD hydrogels and showed insulin secretion capacity up to 100 days (52). Using this system, green fluorescent protein-expressing (GFP-expressing) islets were transplanted into non-obesity diabetes (NOD) mice. During the 9-week period of follow-up, high density GFP signal was detected in recipient mice and significant angiogenesis was observed within the capsules. The reduction in capsule size allowed the graft to be confined to a specific graft site, enabling graft retrievability, reducing long-term complications and providing better long-term function than traditional alginate capsules. It is important to note that capsules implanted in the intraperitoneal space that cannot be removed pose safety concerns due to potential fibrotic reactions or adhesion to vital organs in the abdominal cavity (52). Edward A et al. added VEGF into polyethylene glycol maleimide (PEG-MAL) hydrogels, allowing on-demand VEGF release through enzymatic degradation. Rat islets encapsulated in PEG-MAL hydrogel secreted insulin during culture and were transplanted into the intestinal mesentery of healthy rats, and blood vessels were rebuilt within 4 weeks (53). In comparison with the encapsulation in conventional alginate-based materials, VEGF was released over an extended period of 7 days. Tissue samples were retrieved one week after implantation, and the macroscopic images clearly showed blood vessels extending from surrounding tissue to PEG-MAL hydrogel. Taken together, these results indicated that PEG-MAL could be used as islet transplantation vector and paved the way for the addition of other ECM components in PEG-MAL.

Nanoparticles (NPs) can be defined as particles which are 1–1000nm in size and possess colloidal properties. Enhance the delivery of attached or encapsulated substances by constructing nanoparticles with specific properties and release characteristics. Unlike other encapsulate methods that immobilize the cells or substances to be encapsulated in a micron-sized gel matrix, nanoencapsulation methods are usually based on the formation of nanomembranes around cells or organs (102, 103). Nanoencapsulation is a technology for encapsulating islets through conformal coating (104), mostly relying on the use of a nozzle method (105). As compared with conventional microcapsules, conformational coating allows for the formation of thin-films covering each individual islet (106). Both the size of the resulting materials and the thickness of the film are adjusted to the size and morphology of individual islets. This technology gives rise to nanocapsules (107, 108), for which the thickness of the protecting membrane favors the bi-directional diffusion of oxygen, nutrients and metabolites. Where possible, there is a correlation between the composition, physicochemical properties, frequency and route of nanoparticle implantation into the human body and tissue and blood toxicity, immunotoxicity and genotoxicity (109), and these factors cannot be ignored when designing nanoparticles.

Due to the small size of nanoparticles, they can cross cell membranes and enter blood and organs, and prolonged exposure of the body to these particles can lead to impaired clearance, inflammation and fibrosis (110). Also, the toxicity of the material affects the viability of the cells when the material is encapsuled around them. Toxicity can be categorized as acute (observed within less than 24 hours after single administration) or repeated (administration within 24 hours), subacute (observed within less than 1 month following repeated exposure), subchronic (observed within 1-3 months following repeated exposure) and chronic (observed after 3 or more months of chronic exposure) (109). Smaller nanoparticles have a higher specific surface area and cause larger contact region to cells which resulting in more toxic to cells. Nanoparticles are also easily removed by the body because their small size, which may run counter to our desire to keep encapsulated islets in the body for as long as possible playing their roles. Understanding the relationship between particle volume size and toxicity, clearance rate, degree of inflammatory fibrosis can help predict and design the appropriate size and frequency of implantation (111).

The usual shape of nanoparticles is spherical, which gives little attention to the effect of shape on nanocapsules. However, the shape of the nanoparticles affects the contact area of the material with the cells and the body internal environment, the diffusion distance of the material, the hydrodynamics in the body, etc. Different shapes of nanoparticles (mesoporous silica, long rod, short rod, spherical) were designed and it was found that rod-shaped nanospherical particles stay longer in the gastrointestinal tract than spherical ones. However, during excretion, spherical nanoparticles were cleared more quickly than rod-shaped nanoparticles (112). Therefore, the appropriate nanoparticle shape may be designed to achieve easier substance transport for therapeutic purposes.

In addition, nanoparticles obtain desirable surface chemistry through surface modifications, such as the introduction of PEG (103). Surface charge also affects the biosorption and half-life of nanoparticles (113). It is important to note that the above properties of nanoparticles may change upon entry into the human body (e.g., protein adsorption, etc.) and therefore, data on *in vivo* exposure are important for long-term safety assessment.

Several reports highlighted enhanced islet functionality under those conditions (114).

In clinical islet transplantations, the Edmonton protocol is the most standardized method (5). While the injection of non-encapsulated islets through the portal vein is appropriate, similar protocol applied to encapsulated cells may lead to severe complications, including the formation of blood clots due to the size of the system. In addition, the incomplete protection of the islets may trigger a strong immune response. Glucagon-like peptide-1 (GLP-1) stimulated insulin secretion in response to high glucose levels (54). Several studies pointed toward the reduction of the graft volume by encapsuling the islets within a biotin-PEG-GLP-1 conjugate using the layer-by-layer method, and the reduced size of the transplant was compatible with the Edmonton procedure without blocking the portal vein. Kim et al. developed cell-mimic polymersomes (PSomes) based on PEG-*b*-PLA (poly (ethylene glycol)-*b*-poly (DL-lactic acid)) for the coating neonatal porcine islet-like cell clusters (NPCCs). NHS-PSome-coated NPCCs and non-coated NPCCs were transplanted under the kidney capsule of C57/BL6 mice. The transplanted kidneys were removed after 14 days for immunohistochemical staining and it was found that there was significantly less infiltration of immune cells in the NHS-PSOME-coated group than in the uncoated NPCC group, without affecting the insulin secretion capacity of the coated cells (55). Neocrin Inc. transplanted PEG nano-encapsulated porcine islets under the renal capsule of diabetic rats. There was no significant fibrosis after 2 weeks and viable encapsulated porcine islet tissue existed more than 100 days after transplantation (56).

Despite these promising studies, the translation of islets loaded nanocapsules to the clinics is facing a series of limitations. The ultra-thin membranes may result in partial exposition of the coated islets to the immune system of the recipient, thus impairing their long-term survival. In addition, the retrieval of nanosized capsules remains challenging, which may trigger a series of potential adverse events (115, 116). While reinforcement of the thin-film membrane can be achieved by ultraviolet-light (UV-light) induced photo-crosslinking, such conditions may be detrimental to islet survival (117). Conformal coating, due to its tight attachment to islet cells, may cause the infiltration of the coating biomaterials into islets and their interaction with islets, leading to necrosis (118). Considering the above limitations, further work is needed to develop new methods for safer nanocapsule-based cell therapies (119). For example, the core-shell encapsulation technique has been developed, and several studies have shown that the use of core-shell encapsulation method is beneficial and can improve the cell survival rate by regulating the core materials (120, 121). The islets also have less chance of protruding from the side of the capsule, reducing the adverse immune response (68).

Materials that mimic biological tissues primarily require excellent mechanical properties and long-term resistance to the

formation of fibrosis caused by foreign body reactions. However, these standards are often difficult to achieve within a single material as they would require the combination of both hydrophobic and hydrophilic domains. Zwitterionic hydrogels are particularly promising for solving this problem (**Figure 5**) (122) and were applied to many medical fields. However, existing zwitterionic hydrogels do not have the long-term mechanical stability and antifouling performance needed for cell transplantation applications. Several strategies were developed to overcome the limitation of conventional zwitterionic hydrogels while maintaining their beneficial properties. Liu et al. (57). produced a poly (quaternized triazole carboxybetaine acrylamide) hydrogel which demonstrated reinforced mechanical properties and low non-specific protein adsorption. This material was investigated for the encapsulation of rat islets and further subcutaneous transplantation in immunocompetent diabetic mice. While mice treated with alginate-based microcapsules loaded with similar islet contents became gradually diabetic from day 18 after transplantation, these triazole containing zwitterionic hydrogels triazole moieties maintained normoglycemia for one month in 8 out of the 12 mice treated. In addition, post-retrieval histological analysis of the grafts showed abundant blood vessels and loose fibrotic overgrowth. Interestingly, the formation of blood vessels was significantly decreased for the mice who did not maintain normoglycemia.

COMBINATION OF NATURAL AND ARTIFICIAL HYDROGELS

Hydrogels resulting from natural polymers generally display favorable properties including angiogenesis, antibacterial activity and chemotaxis. Nevertheless, their quality is highly dependent on batch-to-batch variability and their mechanical properties hardly meet the required criteria for efficient islet transplantation. Therefore, the combination of natural and synthetic hydrogels offers the opportunity to correct the defects of natural components while maintaining their beneficial properties.

PLG is composed of copolymer of Lactide and glycolide (Poly (Lactide-co-glycolide). Besides controlling the distribution and density of transplanted islet cells in the scaffold, PLG's dense pores are conducive to substance exchange and vascular reconstruction (123). T cells are the main immune cells responsible for T1D and islet transplantation rejection (124), and Fas ligand (FasL) can induce apoptosis by interacting with Fas on T cells to achieve immune tolerance (125). Co-transplantation of FasL protein overexpressed myoblasts with islets restored euglycemia without continuous immunosuppression (126). This approach has been applied to islet encapsulation. The researchers modified the surface of the pancreatic islets with FasL chimeric with streptavidin (SA-FasL) and combined the modified islets with a scaffold formed by coupling PLG and biotin, and transferred it into the epididymal fat pad of diabetic mice. The implanted islets showed sustained survival after short-term (15 days) immunosuppressive therapy, maintaining normal blood glucose for 200 days (59).

In addition to FasL modification of islet cells, co-embedding with regulatory T(Treg) cells is a novel approach to exploit immunosuppression. The ability of Treg cells to induce immune tolerance (127) provides a good idea for the treatment of autoimmune and alloimmune responses. PLG, as the scaffold for islet transplantation, co-located with Treg cells at the intra-abdominal fat of NOD mice, avoiding the instant blood-mediated inflammation caused by hepatic vein transplantation. This method induced long-term survival of transplanted cells without systemic immunosuppression, and realized the function of maintaining normal blood glucose (60). However, it leads to a non-specific inflammatory response, and the implantation process and biomaterials recruit antigen-presenting cell (APC) *in vivo*, inducing the secretion of inflammatory cytokines at the site of injury.

ANTI-INFLAMMATORY HYDROGELS

Current polymer hydrogel networks have been shown to block immune response cells and antibodies to protect islet cells, but permeation-selective barriers do not prevent low-molecular-weight cytotoxic molecules, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) from diffusing into the capsule material and damaging islet cells (35, 128). Approaches to encapsulate cells and tissues in anti-inflammatory hydrogels can solve this problem. Investigators have demonstrated that the

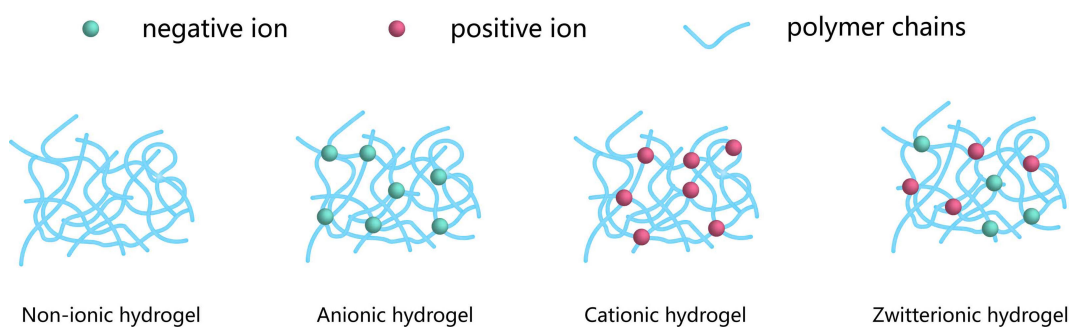

FIGURE 5 | Classification of the encapsulation, according to the network electrical charge. Hydrogels may be categorized into three groups: Nonionic, Ionic (including anionic or cationic) and Zwitterionic. The blue networks are hydrogel polymers, the green spheres are negative ions, and the red spheres are positive ions.

natural polymeric hydrogels can exhibit strong anti-inflammatory activities, make them promising candidates as advanced therapeutics for tissue reconstruction applications.

Tannic acid (TA) is a polyphenolic natural product and an effective antioxidant (107). By using TA, antioxidants and neutral polymer poly(n-vinylpyrrolidone) (PVPON) multilayers to form a nano-thin encapsulation material PVPON/TA. After xenografting, PVPON/TA-encapsulated neonatal porcine islets were not defective in glucose responsiveness and had reduced expression of MHC-II and co-stimulatory molecules CD40, CD80 and CD86 in antigen-presenting cells (129).

Hyaluronic acid (HA) is the main component of the ECM, and the high molecular weight of HA has anti-inflammatory and immunosuppressive properties (130). *In situ* cross-linked hydrogels consisting of hydrazone-cross-linked aldehydes- and hydrazine-modified HAs are effective in preventing peritoneal adhesions in a rabbit side wall defect-cecal abrasion model (131). Cross-linking of dexamethasone modified HA to form an injectable hydrogel reduces TNF-α and IL-6 production by mouse primary macrophages and causes less inflammation compared to unmodified cross-linked HA (132). The Gel/Alg@ori/HA-PEI@siRNA-29a hydrogel prepared by Yang et al. on the basis of HA, by modifying HA, adding ori to shorten the inflammatory period and adding siRNA-29a to promote angiogenesis, was demonstrated in *in vivo* experiments to significantly promote diabetic wound healing and inhibit pro-inflammatory factors (IL-6 and TNF-α) (133).

Modification of artificial hydrogels with anti-inflammatory peptides and adhesion peptides is also a way to prepare anti-inflammatory hydrogels. Immobilization of peptides that inhibit cell-surface interleukin-1 (IL-1) receptors maintain the activity of cells encapsulated in PEGylated hydrogels exposed to a variety of cytokines including IL-1, TNF, and interferon (IFN). These peptide-modified hydrogels effectively protect encapsulated cells from β-cell-specific T cells and maintain insulin release from MIN6 cells (Mouse islet tumor cells) stimulated by glucose (58).

NON-HYDROGELS AS BIOMATERIALS FOR CELL ENCAPSULATION

In addition to hydrogels, some non-hydrogels are also used as capsule materials, such as some biological cells and tissues, or some non-degradable synthetic polymer materials (**Table 2**).

In order to mitigate the adverse inflammatory response caused by the implantation of biomaterials, islet grafts were coated with leaving cells to improve their host biocompatibility. For instance, islet surface was covered with a biotinylated PEG-lipid layer and further conjugated with streptavidin-modified HEK293 cells (human endoderm kidney cell line) (134). The cell layer formed on the surface of the islets will be the immune barrier membrane. Such surface modification reduced the incidence of cell necrosis and maintained persistent glucose-stimulated insulin secretion ability of the protected islets.

Transplantation of islets to an extravascular site may not cause immediate inflammation, but on the other and, the revascularization process may also cause graft loss. Therefore, it is important to find the balance between angiogenesis and graft hypoxia. For these reasons, various human tissues have been combined with transplanted cells, such as the human amniotic membrane (HAM) and human amniotic epithelial cells (HAECs). Amniotic membrane consists of the epithelial layer and vascularless matrix offering an optimal scaffold for islet cells (135, 137). HAECs have a number of stem cell properties, such as potentially inducing angiogenesis (138) and being induced to differentiate into insulin-producing cells (139). Wanxing Cui et al. (135) placed decellularized 1.0x1.0cm^2 HAM on the surface of the transverse membrane of the left lobe of the liver. Human islets and HAECs were mixed in Hank and the mixture was injected into the space between the HAM and the liver surface with a 200μL pipet tip. By the third day after transplantation, four of the seven recipient diabetic mice had normalized the blood glucose, and after two weeks seven recipient mice (100%) became normoglycemic.

A novel islet encapsulation silicon device, "NanoGland", consists of an outer membrane with parallel nanochannels (3.6–40 nm) and perpendicular microchannels (20–60 microns) surrounding islets. The nanochannels are designed to provide immunoprotection and the microchannels are thought to enhance the engraftment. In addition to maintaining its own flexibility, the material still has precise nanoscale pores. Mice transplanted with allogeneic islets encapsulated whit the device showed function for up to 90 days and subcutaneous implantation of the NanoGland with human islets in mice showed survival of implants for more than 120 days. Analysis of the tissue surrounding NanoGland showed the presence of blood vessels, as well as the typical signs of fibrosis (136).

CONCLUSIONS AND PERSPECTIVES

The current methods for cell encapsulation include various approaches, such as nano-, micro-, macroencapsulation and numerous natural, bio-inspired and synthetic polymers have

TABLE 2 | Non-hydrogels as biomaterials for cell encapsulation.

Material	Honor	Recipient	Graft	Site	Result	Reference
HEK293	Syrian hamsters	_	islets	_	no central necrosis and well glucose stimulation (5d)	(134)
Human amniotic membrane (HAM)	Human	Severe combined immunodeficiency mice	2,000 IEQ of human islets mixed with 0.4x 10^6 HAECs	surface of the liver	all recipient attained euglycemia (by 2 wks)	(135)
NanoGland (Silicon)	Human	Nude mice	islets	subcutaneous	islet viability and responsiveness (120d)	(136)

been tested. Although significant progress has been achieved and some preclinical trials have been performed, important hurdles still remain.

This article focuses on islet encapsulation, either by co-encapsulation or modification of the encapsulated material, in order to reduce the attack of the immune system on the graft and to maintain the cellular activity and physiological function as much as possible. Due to various constraints, islet capsule transplantation has also developed an increasing number of contents as well as material modifiers. For example, due to the shortage of donors, attention has been turned to xenotransplantation, such as exploring the transplantation of neonatal porcine islets. Neonatal porcine islets need to be cultured *in vitro* for four weeks to reach the same level of insulin secretion as adult islets (140). However, compared to mature porcine islets, neonatal islets are more likely to build up immune tolerance in the recipient, making them less susceptible to immune attack after maturation.

Therefore, the design of therapeutic hydrogels was envisioned. Currently this multifunctional hydrogel has been applied to the biomedical field, most commonly in multi-stage drug delivery (141), which is a new promising direction for future cellular encapsulation. The functional hydrogel assembles insulin and neonatal porcine islets into a system. After implantation into human body, the insulin in the early system is released slowly until the porcine islets mature. At later stage, hydrogel protects islets from immune attack. Free passage of nutrients and metabolites through the envelope, the mature pig islets secrete insulin at a later stage to achieve the capsule function. This multifunctional programmed hydrogel can be applied to various cells such as differentiating stem cells, etc., opening up a new avenue for the treatment of T1D.

The major limitations for large clinical application include the great variability of biomaterials, with insufficient biocompatibility leading to some degree of foreign body reaction and progressive fibrotic reactions. Based on previous studies that generally used one or two combined strategies to protect islet graft function, a multifunctional encapsulated hydrogel model with multiple functions is the way forward for our development.

With the continuous progress of technology, additional modifications of polymers should achieve higher degree of biological compatibility

AUTHOR CONTRIBUTIONS

LB and YW conceived and designed the review. QZ, CG-G, and YL wrote the manuscript. QZ and ZG prepared the figures. LB, YW, and SG-L reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Engineering Islets From Stem Cells: The Optimal Solution for the Treatment of Diabetes?

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Diabetes is a metabolic disease characterized by insulin deficiency. Bioengineering of stem cells with the aim to restore insulin production and glucose regulation has the potential to cure diabetic patients. In this review, we focus on the recent developments for bioengineering of induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), and pancreatic progenitor cells in view of generating insulin producing and glucose regulating cells for β-cell replacement therapies. Recent clinical trials using islet cells derived from stem cells have been initiated for the transplantation into diabetic patients, with crucial bottlenecks of tumorigenesis, post-transplant survival, genetic instability, and immunogenicity that should be further optimized. As a new approach given high expectations, bioengineered islets from stem cells occupies considerable potential for the future clinical application and addressing the treatment dilemma of diabetes.

Keywords: stem cell, islet, diabetes, engineering, therapeutic efficacy

INTRODUCTION

Diabetes is one of the major public health challenges of the 21st century and bears heavily to global health costs. An estimated number of 463 million adults (1 in 11) around the world are living with diabetes, and this number is projected to reach 700 million by 2045 (1). Type 1, type 2 and gestational diabetes mellitus are three main categories of diabetes, and type 2 diabetes remarkably accounts for around 90% of diabetes cases worldwide (1). Although lifestyle modification and pharmacotherapy are both efficient to treat type 2 diabetes, marked variability in outcomes still widely exists resulting in irregular monitoring, sub-optimal use of effective medicines and inevitable disease progression due to decline of β cell function. Therefore, innovative therapies are required to implement for delaying β cell lost, regeneration of endogenous β cell mass or replenishment of β cells with engineering islets from stem cells.

Stem cells are undifferentiated cells with self-renewal and differentiation into various cell types (2–4). Since 1960s, scientists have successively identified and isolated hematopoietic stem cells (HSCs), bone marrow stem cells (MSCs), embryonic stem cells (ESCs) and developed induced pluripotent stem cells (iPSCs) (5). Thereinto, adult stem cells such as HSCs and MSCs are derived from bone marrow, skeletal muscle, fat, amniotic fluid, umbilical cord blood, skin, placenta and other tissues or organs, while embryonic stem cells are derived from embryonic tissues (6–9). IPSC are obtained through genetic reprogramming of somatic cells by ectopic expression of four transcription factors (OCt3/4, SOX2, C-MyC and Klf4) and have been generated from somatic cells such as mouse embryonic fibroblasts (MEF), adult mouse tail fibroblasts as well as human fibroblasts (10, 11) (**Figure 1**). Importantly, embryonic stem cells are totipotent and can differentiate into cell types derived from all three germ layers (12, 13). In contrast to embryonic stem cells, multipotent adult stem cells have limited self-renewal abilities and are prone to differentiate into specific adult tissue cells such as adipose tissue and muscle tissue (4).

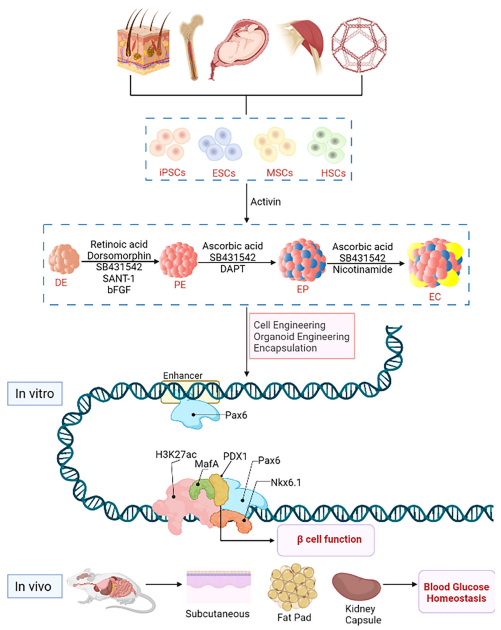

FIGURE 1 | Stem cell engineering for type 1 diabetes. Stem cells originate from various sources, e.g. embryonic stem cells with strong ability of differentiation and self-renewal from embryonic tissues, hematopoietic stem cells and bone marrow stem cells from tissues or organs such as placenta, amniotic fluid, umbilical cord, bone, skeletal muscle, fat and skin; induced pluripotent stem cells are produced by genetic reprogramming of somatic cells. *In vitro*, predicted paired box 6 (PAX6) activates the expression of pancreatic β-cell-specific genes and proteins, such as pancreatic duodenal homeobox factor-1 (PDX-1) and NK6 Homeobox 1, (NKX6-1) and musculoaponeurotic fibrosarcoma oncogene family A (MafA), and interacts with these factors at the protein level to promote β-cell function. *In vivo*, hypoglycemic homeostasis is rapidly reestablished in diabetic immunodeficient mice (NOD/SCID) after transplantation with subcutaneous, renal capsule or fat pad.

With the continuous exploration of stem cells, an abundance of studies identified that cells such as MSCs and ESCs can grow indefinitely outside the body and maintain their ability to differentiate, which highlight their potentials as alternative sources of organ and tissue replacement (4, 14–16). Encouragingly, the results of emerging preclinical studies and clinical trials for diseases, such as diabetes have deepened our understanding of the use of stem cells in tissue engineering and cell therapy (17, 18). To date, new progress has been made in the treatment of brain diseases such as cerebral palsy (19–23), stroke (24–28), blood diseases (29), eye diseases (30–32) as well as diabetes (33–36), and the exploratory research on the treatment of diabetes with stem cells is developing in the right direction (36). In this review, we summarize the research field for stem cell differentiation and islet engineering, emphasizing on the efficacy of this new bioengineering technology applied for diabetes care, and elucidate current breakthroughs and future challenges of stem cell differentiation into islets.

iPSCs-DERIVED ISLET CELLS FOR ISLET REPLACEMENT THERAPIES

In recent years, it has been demonstrated that iPSCs have unlimited self-renewal ability and can be differentiated into multiple cell types such as neural stem cells (NSCs) (37), cardiomyocytes (38), dopaminergic neurons (39) and hepatocellular like cells (40). IPSC-derived islet cells might constitute a new source for islet cell replacement therapies (41). *In vitro*, most iPSC-derived cell lines initially express pancreatic and duodenal homeobox 1 (PDX1) and then further differentiate into PDX1, glucose transporter 2 (Glut2), musculoaponeurotic fibrosarcoma oncogene family A (MafA) and insulin expressing end-stage cells. One specific iPSCs cell line was detected to first express SOX17 and gradually express the β-cell-specific marker SOX9, PDX1 at later stages. The co-expression of C-peptide and PDX1 at a final stage confirmed the differentiation into insulin-producing cells (42). Additionally, it was shown that in order to obtain insulin-secreting cells *in vitro*, factors such as retinoic acid (RA), glutamine, noggin, nicotinamide and growth factors such as keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) are essential for the directed iPSCs differentiation (42, 43). Therefore, owing to the tremendous research potential of iPSCs in β cell replacement therapies, these stem cells are promising for further drug development and transplant medicine applications.

Furthermore, tissue engineering techniques such as 3D bio-printing have been substantially evolved since the 1980s for human therapeutic applications, including the creation of a bio-artificial pancreas. 3D bio-printing involves the isolation and expansion of human cells, followed by the automated printing of biodegradable scaffolds containing such cells. 3D bio-printed scaffolds are under investigation for various applications such as therapeutic devises, and *in vitro* model systems for analyzing diseases or screening drugs (44, 45). Undoubtedly, 3D bioprinting and regenerative medicine cooperatively hold great promise in building and assembling a bioartificial pancreas.

Organoids are defined as 3D multi-cellular spheroids obtained in *in vitro* cultures. Numerous 3D cell culture methods using islet cells had been described to obtain hetero cellular islet organoids (46). Remarkably, such hetero cellular islet organoids may integrate different types of supporting cells, such as endothelial cells, into insulin-producing structures, which is a valuable strategy to increase neovascularization of transplanted islets (46). Therefore, using human pluripotent stem cells to create organoids that resemble human pancreatic islets *in vivo* could help to overcome the organ scarcity. Herein, Tao et al. produced human islet organoids from human iPSCs using a perfusable organ on-chip system. The system integrated functional β-cells obtained after induction of endoderm, followed by differentiation and amplification of pancreatic progenitor cells and maturation of endocrine cells (**Figure 2**) (47, 48). As described in **Figure 2**, the islet-like organ was generated by step wise incubation with essential differentiation factors. Starting from primary embryoids (EBs), endodermal production was induced by activin, pancreatic final endodermal production was induced by dorsomorphin and RA, and finally the insulin producing pancreatic β-cell was induced by nicotinamide. This islet-like organ was generated under dynamic perfusion system, a multilayered microfluidic device composed of four parts top and bottom polydimethylsiloxane (PDMS) layers, through-hole PDMS membrane and polycarbonate porous membrane as separators (49). Fresh media was pumped through the upper and lower layers at 100μl per hour thereby providing continuous supply of media and nutrients for the formation and long-term culture of islet organs after EBs formation. In addition, produced islet-like organs contained heterogeneous islet-specific α- and β-like cells with sufficient cell viability. Simultaneously, during culture expression of pancreatic β-cell-specific genes and proteins such as PDX1 and NK homeobox 1 (NKX6-1), and C-peptide proteins related to insulin secretion were increased. Together, these results provide evidences that an islet-like organ generated through a perfusable islet-on-chip system is similar to the reproduction and development of human islets. And this technique offers a feasible and effective engineering method for generating functional islet-like organoids derived from iPSCs in a bionic microenvironment. Moreover, Yoshihara' team also found that human iPSC-derived human islet-like organoids can rapidly restore glucose homeostasis after transplantation in diabetic immunodeficient mice (NOD/SCID) (50). The expression of immune checkpoint protein programmed death ligand 1 (PD-L1) in organoids was restored and glycemic homeostasis was maintained during 50 days in immunocompetent diabetic mice. Further, interferon-γ *ex vivo* exposure of human islets as well as human islet-like organoids derived from iPSC induced strong endogenous PD-L1 expression. Transplantation of PD-L1-overexpressing islet-like organoids into immunocompetent mice showed that iPSCs were protected from graft rejection in both allo- and xenotransplant settings. However, no studies on the transplantation of PD-L1-overexpressing islet-like organoids generated from iPSCs in the setting of the human immune system have been reported, which indicates the lack of evidence on determining PD-L1 expression profile for protecting human cells against the allogeneic human immune system. Meanwhile, this vacancy may greatly stimulate the progress of related research topics.

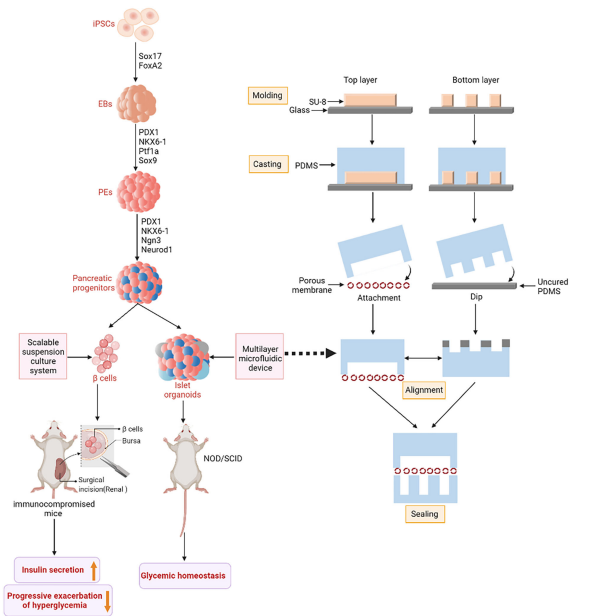

FIGURE 2 | IPSCs-derived islet function replacement. iPSCs differentiate into insulin-secreting cells under the action of related transcription factors. Embryoid bodies (EBs) are formed under the influence of SLEy-related high mobility group box 17 (SOX17) and Forkhead box A2 (FoxA2). Pancreatic and duodenal homeobox 1 (PDX1), NK6 Homeobox 1 (NKX6-1), Pancreas Associated Transcription Factor 1a (PTF1a) and SOX9 guide to pancreatic Endoderms (PEs). Finally, PDX1, NKX6-1, Neurogenin 3 (Ngn3) and Neuronal Differentiation 1 (NeuroD1) differentiated into pancreatic progenitor cells. Pancreatic progenitor cells are directed to produce glucose-responsive β-cells. The figure on the right shows the fabrication method of microfluidic device. SU-8 photoresist is rotated onto two clean glass wafers and then selectively cured under ultra violet (UV) light using different masks. The mixture of Polydimethylsiloxane (PDMS) monomer and hardener then produces a two-layer copy of PDMS. Finally, the two PDMS replicas are sealed together with an intermediate porous membrane.

Another previous study described that glucose-responsive β-like cells can be efficiently produced by a scalable suspension culture system from ESCs and iPSCs *in vitro* (51). These stem cell-derived β-like cells (SC-β) expressed cytoplasmic C-peptide and nuclear protein NKX6-1, which is similar to islet β-cells. Other studies that transplanted human SC-β-cells into immune-compromised mice also showed β-cell functionality *in vivo*. Glucose challenges appeared after SC-β transplantation and human insulin in the blood was measured within weeks after transplantation in mice (17, 52, 53). Consequently, the cell transplantation under the renal capsule of immune deficient mice rapidly reversed the progressive exacerbation of hyperglycemia. Besides, 18 weeks after transplantation, it was remarkably observed that the mice receiving SC-β maintained normal human insulin secretion. Thus, SC-β transplantation successfully improved hyperglycemia in diabetic mice. As an

advantage for clinical application, SC-β-cells can be produced from iPSCs of patients to avoid allogenic rejection after transplantation (51). In the future, autologous SC-β cell transplantation in combination with Treg adoptive immunotherapies may selectively suppress autoimmunity in patients with type 1 diabetes mellitus (T1DM), which could eliminate major obstacle for the cure of T1DM (54–56).

Despite the potential of iPSC to develop into a therapy for diabetic patients, important issues still need to be solved. The current limitations include: low reprogramming efficiency, reprogramming factors related to tumorigenesis, low survival and engraftment, loss of cell phenotype after transplantation, genetic instability, epigenomic instability and inherent immunogenicity need to be considered (57–61). Recently, a clinical case was reported describing the development of a teratoma in a diabetic patient after iPSCs-derived β cells transplantation (62). In this patient, β cells differentiated from autologous iPSCs were initially injected into the deltoid muscle where a mass with enlarged axillary lymph nodes were detected at two months after implantation. This tumor was characterized by rapid growth, local lymph node metastasis, more cellular atypia, and chemotherapy-resistance (62). As a milestone in regenerative medicine, iPSCs also shed new light on the treatment of age-related macular degeneration (AMD), one of the main causes of irreversible blindness. The researchers used the retinal pigment epithelium (RPE) cell sheets induced by autologous iPSCs from 2 patients with AMD through subretinal surgery. As a result, no serious adverse event was observed in 25 months of follow-up and no signs of rejection was noted without the administration of immunosuppressants in one patient, which provided valuable information on the feasibility and safety of iPSCs in the treatment of patients with macular degeneration and created a precedent for the clinical transformation of iPSCs in the field of regenerative medicine (63). *In vivo* tumorigenicity tests and a series of genomic analyses were both performed, although iPSCs-derived RPE cells had a low proliferation rate. In the iPSC-derived RPE cells obtained from Patient one, no genomic aberrations that were suggestive of tumorigenicity was found. However, three aberrations in DNA copy number (deletions) in iPSCs obtained from Patient two were detected, which could affect expression of genes encoded by both the deleted DNA and by DNA flanking the deletions (63). Based on the published information, the possible influence of these alterations on tumorigenicity could not be determined. In the reprogramming process to pluripotency and the cultivation of iPSCs, genetic instability was reported to be enhanced, potentially leading to additional genomic abnormalities (64). These genetic changes could negatively influence the performance and functional activities of iPSCs and increase tumorigenicity in replacing damaged tissues (65). Therefore, genomic stability must be maintained after reprogramming for further clinical uses.

As a renewable source of autologous cells, iPSCs have great prospects in regenerative medicine. It was generally accepted that autologous cells should be immune-tolerated by the recipient from whom the iPSCs are derived, whereas accumulating evidences remarkably revealed the rejections from autologous iPSC-derived cells, although the underlying related mechanisms remained controversial and still in the process of being gradually defined. As mentioned above, RPE cells derived from autologous iPSCs made the remission of AMD achievable. Even in non-ocular locations, they are also immune tolerated. However, smooth muscle cells (SMCs) produced from autologous iPSCs appeared to be significantly immunogenic, partly result from the abnormal expression of immunogenic antigens in iPSCs-derived SMCs (66). In C57BL/6 (B6) mouse transplantation model, immunogenic antigen-expressing B6 iPSCs and their differentiated target cells were immune tolerated under the kidney capsule but immune rejected when transplanted subcutaneously or intramuscularly owing to the lack of functional antigen presenting cells, indicating that the immune response toward antigens was also dependent on the immune environment of the transplantation site (67). Furthermore, autologous iPSCs and their derivatives were not inherently immunologically inert for autologous transplantation, due to *de novo* mutations in mitochondrial DNA (mtDNA) probably produced in the process of reprogramming to the iPSCs stage, long-term culture and differentiation into target cells. And these mtDNA mutations could encode neoantigens and elicit highly specific immunological response based on the host's major histocompatibility complex genotype, which implied the indispensability to recheck the mtDNA mutations iPSC-derived products (68). Aberrant gene expressions in some cells generated from iPSCs can cause T cell-dependent immunological response in syngeneic recipients (69). Therefore, the immunogenicity of therapeutically valuable cells produced from patient-specific iPSCs should be assessed before clinic application in patients. In order to adopt the optimum immunosuppressive strategy to allow their engraftment, detailed evaluation of the inherent immunogenicity profiles of iPSC-derived somatic cell lineages is considerably required.

ESCs-DERIVED β-CELLS FOR INSULIN SUPPLEMENTATION

Insulin-secreting cells derived from pluripotent embryonic stem cells (ESCs) have emerged as one of the most attractive therapeutic alternatives for diabetes (70). Recently, it was shown that after transplantation of *in vitro*-differentiated stem cell-derived islets into immune-compromised mice, islets acquire a mature β-cell gene expression profile and can control blood glucose in the long term (71). Moreover, under adherent and suspension culture conditions, ESCs spontaneously differentiated into insulin-secreting cells *in vitro* with a very high proportion as observed through insulin immunohistochemical staining (72). A refined method for generating more mature insulin-producing cells from human ES has been described by Wang et al. using a three-dimensional differentiation culture. Most important steps for ES differentiation into insulin-secreting structures were first outlined by Lumelsky et al. (73) (**Figure 3**). These three-dimensional clusters were similar in structure to normal islets and contained all cell types

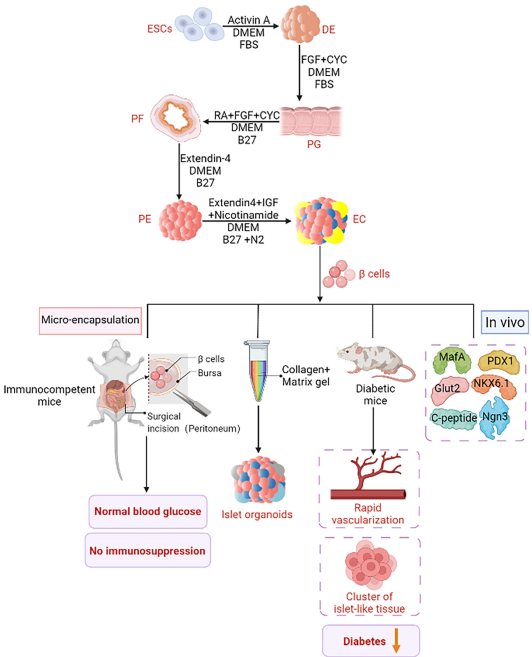

FIGURE 3 | ESCs derived insulin supplement. An improved ESCs pancreatic differentiation protocol was developped. First, under the action of Activin A, DMEM, and FBS, one definitive endoderm (DE) was established through mesoderm. In the second step, activin A was removed and kaad-cycloamine (CYC) and fibroblast growth factor (FGF) were added to facilitate the transition from DE to the primitive gut tube (PG). The third step is to add retinoic acid (RA) and B27, and then to the posterior foregut endoderm (PF). Step 4: Extendin 4 (EX4) was added to support pancreatic lineage specific differentiation. The fifth step is to generate endocrine cells under the stimulation of EX4, IGF (insulin-like growth factor) and nicotinamide. These cells differentiate *in vitro* into insulin-secreting cells, Pancreatic and duodenal homeobox 1 (PDX1), neurogenin 3 (Ngn3), MAF BZIP Transcription Factor A (MafA), glucose transporter 2 (Glut2), NK6 Homeobox 1 (NKX6-1) and C peptide were expressed. When these cells are injected into diabetic mice, diabetes is effectively ameliorated by rapid vascularization and the formation of a cluster of islet organoids. Islet organoids are obtained from ESCs by combining collagen and matrix gel in the biomimetic 3D scaffold. Islet sacking can quickly restore normal blood glucose without immunosuppression.

of the endocrine pancreas (73). *In vitro* studies revealed that glucose as well as various secretagogues could trigger insulin release from these structures through similar mechanisms as in human islets. After transplantation into diabetic mice, these insulin-producing cells underwent rapid vascularization and formed clusters of islet-like tissue. Further, others showed that transplantation of islet cell clusters at stage 4 of differentiation, enriched of NKX6-1-expressing pancreatic progenitor cells through the action of a PKC activator, accelerates the maturation of insulin-secreting cells *in vivo*. Gradually, differentiation of human embryonic stem cells into islet cells has been widely used (74–76).

Solid evidence unveils that extracellular matrix (ECM) not only provides structural information for cells, but also plays a guiding role in cell development, which is crucial for maintaining tissue homeostasis and of great significance in embryogenesis, tissue-specific development and stem cell differentiation (77, 78). Cell-stromal interactions can promote β-cell proliferation (79, 80), insulin secretion (81, 82) and islet development (83, 84). Oberg-welsh and his group have demonstrated that ECM significantly enhances insulin secretion in fetal pig islet-like cell clusters *in vitro* (85). Besides, islet-like organs derived from human embryonic stem cells were successfully developed in a biomimetic 3D scaffold by combining collagen with matrix gel (86, 87). The resulting cell clusters included pancreatic α, β, and pancreatic polypeptide (PP) cells, but most of the resulting islet cells did not express glucagon, somatostatin, or PP. Expression of mature β-cell-marker genes such as PDX1, neurogenin 3 (Ngn3), insulin, MafA and Glut2 was detected in these 3D-induced cell clusters, whereas PDX1, NKX6-1 and C-peptide was highly expressed. Additionally, insulin secretory granules, indicating mature β-cells, were also detected. Although, neither collagen nor matrix gel materials are approved for clinical use, the results shed the light on the feasibility of generating islet-like organs from ESCs. Future breakthroughs by using supporting materials may lead to further progress.

A new transplantation strategy was proposed by Song and Millman who developed a large-pore recyclable 3D printing device composed of biocompatible poly-lactic acid (PLA) for subcutaneous transplantation of SC-β-cells (88). Clusters of SC-β-cells derived from human embryonic stem cells were embedded in biodegradable fibrin gel and inserted in the device. Severe transient hypoxia within the device that occurred after transplantation was mitigated by finite element modeling of cell oxygen concentration and evaluation of oxygen diffusion in different sized cell clusters embedded in hydrogel slabs. These adaptations allowed the device to be operated at physiological oxygen levels. After subcutaneous transplantation of the device into immune-compromised mice, SC-β-cells containing device was found to function for 12 weeks. Retrieved devices were structurally intact. Despite the observed host-tissue invasion, the mechanical strength and recyclability of such a device represent a considerable progress in the field. Other previous methods transplanting islets or cell clusters encapsulated in semipermeable microcapsules composed of alginate are challenged similarly by pericapsular fibrotic overgrowth (PFO) of microcapsules and the difficulty to retrieve grafts (89–91). Therefore, such a retrievable devise is promising for the application of SC-β-cells in regenerative medicine and serves as a platform for future transplantation strategies.

Insulin-producing cells derived from stem cells can address organ donor shortage, while cell encapsulation can reduce or eliminate the need for immunosuppression, minimizing the risks associated with islet transplantation procedures (92–97). Islet encapsulation provides a physical semi-permeable barrier not only preventing immune cell infiltration but also allows diffusion from necessary smaller molecules such as oxygen, nutrients, glucose, and insulin through the microcapsule. This is crucial for achieving widespread clinical use of the technique (98–100). Therefore, transplantation of microencapsulated stem cell-derived islets may extend islet transplantation to a larger cohort of patients. The embedding of immature β-cells derived

from human embryonic stem cells into a sodium alginate hydrogel alleviated the response to foreign bodies *in vivo* and rapidly established normal blood glucose for 25 weeks after its transplantation into the peritoneum of immunocompetent mice (101). Vegas et al. reported long-term glycemic correction using human SC-β-cells in an animal model of diabetic immunocompetent mice (102). SC-β-cells coated with alginate derivatives and intraperitoneally implanted into streptozotocin (STZ)-treated C57BL/6J mice corrected blood glucose levels for 174 days during follow up. Retrieved implants still contained viable insulin-producing cells and showed minimal fibrotic overgrowth. In addition, a novel encapsulation approach was reported by the group of Alice A Tomei, in which transplantation of conformal coated islets, from fully MHC-mismatched Balb/c mice, achieved long-term (>100 days) survival after transplantation into epididymal fat pad or mammary fat pad of diabetic immune competent C57BL/6 mice (103, 104). Conformal coating minimizes capsule thickness, complies with islet shape, reduces transplant volume compared to encapsulated islets and allows glucose stimulated insulin release *in vitro* without delay (105). Moreover, when SC-β-cells were transplanted into gonadal fat pad of diabetic immunodeficient NOD-SCID mice, it was found that both uncoated and conformal coated SC-β-cells reversed diabetes. Blood glucose levels were maintained at normal levels for more than 80 days as obtained with human islets, demonstrating thereby safety and efficacy of this β-cell replacement strategy (103). To date, a small number of encapsulation systems have been used clinically with obvious safety (106). For example, βAir devices are designed for clinical use to ensure oxygen levels necessary for maximum islet function while microcapsules remain in the body. The device consists of two main components, an alginate saline gel plate containing islet modules and a gas chamber. After subcutaneous implantation, islets are oxygenated daily. Islet cells in the lumen absorb oxygen through diffusion *via* the permeable membrane (107). Remarkably, one case report describes a patient whose islets remained fully functional during a 10-month study period (108). Apart βAir devices, Theracyte and Sernova Cell Pouch also offers clinical devices which allows for pre-vascularization prior to implantation (106, 109).

In addition, Vertex Pharmaceuticals Incorporated recently announced unprecedented and positive Day 90 data for the first patient achieving successful engraftment and substantial improvement of islet cell function from VX-880, an novel investigational embryonic stem cell-derived and fully differentiated pancreatic islet cell replacement therapy for the treatment of type 1 diabetes (110). In this Phase 1/2 clinical trial, VX-880 was generally well tolerated with significant improvements in multiple measures, including fasting and peak stimulated C-peptide, HbA1c, and daily exogenous insulin requirement dose (111, 112). These unprecedented results introduce a potentially transformative medicine and deliver a life-changing therapy for T1DM patients and confer the remarkable promotion on the following VX-880 clinical studies, although there are still uncertain problems that need to be further clarified, including unexpected side effects, whether the treatment effectiveness would last a lifetime, and whether repeated treatment would be necessary.

MSCs AND ISLET CO-TRANSPLANTATION FOR ISLET FUNCTION PROTECTION

Transplantation of microencapsulated islets has been extensively studied as a promising treatment for type 1 diabetes. Meanwhile, challenges remain especially in achieving long-term function and reducing inflammation at the graft site that would lead to early islet dysfunction. Further, insufficient angiogenesis around graft sites remains a major issue resulting in malnutrition and hypoxia of encapsulated islets (113, 114). On the other hand, although iPSCs and ESCs have received sustained attention over the years, their clinical transformation is still hampered by ethical issues and risk of teratoma formation (115, 116). Thus, other feasible stem cells demand further investigation to overcome the obstacles mentioned above. Bone marrow mesenchymal stem cells are multipotent stem cells, which are mainly used for cell and regenerative therapy (117) (**Figure 4**). MSCs can secrete various immunomodulatory molecules, such as leukemia suppressor factor (LIF) (118), prostaglandin E2 (PGE2) (119), tumor necrosis factor (TNF)-stimulated gene 6 protein (TSG6) (120), and inhibit the infiltration of macrophages, neutrophils and monocytes into inflammatory sites by the release of TSG6. Besides, MSCs can restrict the fibrotic response by reducing myofibroblast differentiation and ECM deposition in fibroblasts and epithelial cells (121). In addition, MSCs also secrete angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and transforming growth factor-β (TGF-β) (122). These secreted factors support islets to build their own vascular system. Thus, these characteristics confer MSCs the potential for supporting various functions when co-transplanted with islet cells. Earlier studies have demonstrated that MSCs and islet co-transplantation protect islets from problems associated with instant blood-mediated inflammatory response (IBMIR) and fatigue, diarrhea, and immune pneumonia caused by long-term use of immunosuppressive therapy (123–127). In the MSCs and islet co-transplantation, MSCs produce a microenvironment conducive to islet repair and longevity *in vitro* (128), and promote insulin secretion (129) and islet transplantation results in STZ-induced diabetic mice (130). Therefore, MSCs seem to be ideal supporting cells for co-transplantation with islets, although there are still research gaps to be filled soon.

As a common scaffold in tissue engineering, 3D cell culture system and hydrogel compositions can be applied to mammalian cells, such as islets. The small pore size of hydrogel prevents immune cells from passing through, thus protecting the islet from immune rejection, promoting the exchange of oxygen and nutrients, and improving the results of islet transplantation (131, 132). Some scholars embedded adipose tissue-derived mesenchymal stem cells (AT-MSCs) and islets into maleimide-dextrolic anhydride polymer hydrogels to evaluate the therapeutic effect of AT-MSCs in hydrogel composites on type

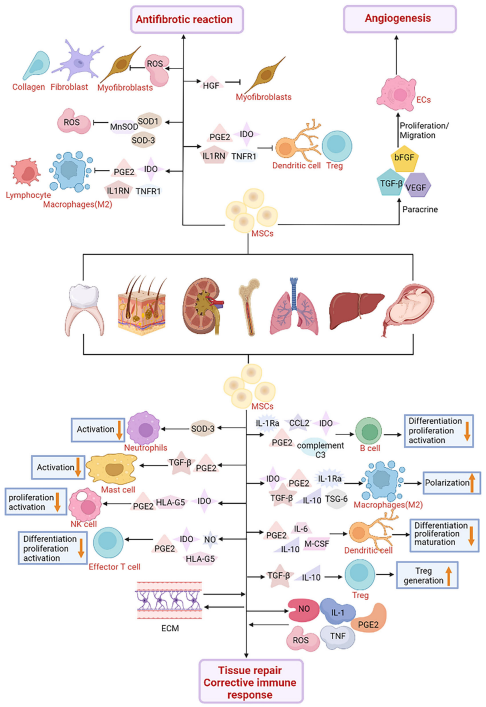

FIGURE 4 | The role of Mesenchymal Stroma Cells (MSCs) in cell engineering technology. MSCs are used in cell therapy and might play a role in the treatment of diabetes. MSCs interact with immune cells such as natural killer cells (NK), macrophages, neutrophils, mast cells and dendritic cells. MSCs secrete a variety of soluble factors, Indoleamine 2, 3-dioxygenase (IDO), prostaglandin E2 (PGE2), C-C motif chemokine Ligand 2 (CCL2), IL-1 receptor antagonist (IL-1RA), complement C3, Tumor necrosis factor- (TNF) stimulated gene-6 (TSG-6), Transforming growth factor β (TGF-β), interleukin-10 (IL-10), interleukin-6 (IL-6), macrophage Colony stimulating factor (M-CSF) and human leukocyte antigen-G5 (HLA-G5) can inhibit the differentiation, proliferation and activation of various immune cell subgroups. Furthermore, MSCs interact with inflammatory factors including nitric oxide (NO), PGE2, tumor necrosis factor (TNF), reactive oxygen species (ROS), interleukin-1 (IL-1) and extracellular matrix (ECM) to promote tissue regeneration, repair and correct abnormal immune responses. In terms of the regulation of fibrosis microenvironment, MSCs release IDO, PGE2, interleukin-1 receptor antagonist (IL1RN), tumor necrosis factor receptor-1 (TNFR1) and other immunosuppressive factors to inhibit the activation of immune cells and the infiltration of inflammatory sites, and promote the formation of regulatory T (Treg) cells. In addition, MSCs produce antioxidant enzymes such as superoxide dismutase 1 (SOD1) and 3 (SOD3) and manganese superoxide dismutase (MnSOD) to reduce oxidative stress and reactive oxygen species levels. In addition, MSCs also secrete hepatocyte growth factor (HGF) and PGE2 in different ways to promote myofibroblast apoptosis and inhibit fibroblast proliferation and collagen production, respectively, thereby inhibiting the fibrotic response. It has also been found in diabetes studies that MSCs can secrete vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), TGF-β and other angiogenic factors. Promotes the formation of new blood vessels and protects the islets from blood-mediated inflammatory response (IBMIR).

1 diabetic mice (133). *in vitro* experiments revealed that AT-MSCs significantly increased insulin secretion. After transplantation, blood glucose dropped from more than 400mg/dl to less than 150mg/dl within 4 days and remained stable until day 32, indicating prime results in treating type 1 diabetes.

A 3D structured cell transplantation platform called *CellSaic* was reported recently and it consisted of cells and petaloid pieces of a medical recombinant peptide (RCP) (134). Unlike traditional animal collagen, these petaloid-shaped pieces increased the surface area for cell adhesion and maintained empty spaces within the scaffold, which permitted substance-diffusion within the scaffold-cell aggregates. The vascular-inducing effect of MSC-*CellSaics* occured through altered release of various cytokines and growth factors, such as interleukin-8 (IL-8), bFGF and VEGF (135). Therefore, Kogawa's team compared graft survival among three transplant conditions, islets, microencapsulated islets and microencapsulated combined with MSC-*CellSaics*. Transplant material remained in a mesh bag under the skin of diabetic mice until recovery (135). During 4 weeks following transplantation, blood glucose levels were significantly reduced and no inflammatory response was observed around the mesh bag within 14 days after transplantation in the MSC-*CellSaic* combined transplantation group compared with other groups. Thus, MSC-*CellSaics* could inhibit inflammation and immune rejection in early transplantation. Co-transplantation of MSC-*CellSaics* with encapsulated islets might be a more efficient approach to increase vascularization of grafts and mitigate inflammatory rejection of microencapsulated islets.

There is increasing evidence that layered slices of cells to construct 3D functional tissue through tissue engineering techniques can help maintain cellular function with nutrition (136). Herein, Hirabaru's team used MSC-sheets as a support for subcutaneous islet transplantation into STZ-induced diabetic SCID mice. Only mice transplanted with islets cultured on MSC-sheets showed normalized blood glucose levels for at least 84 days after transplantation and increased neovascularization compared to islets grafted alone (137). Therefore, this new technical approach using MSC-sheets demonstrated a protective effect on islet survival and function.

Reconstructing a favorable microenvironment allowing integrin interactions could improve the survival rate of isolated islets (129). For instance, the addition of tripeptide arginine glycine aspartic acid (ARG-GLy-ASP, RGD) to microcapsules improves viability and function of C2 and C12 myoblasts (138). Based on these evidences, Laporte et al. developed a biocompatible composite capsule by combining M-rich alginate, RGD G-rich alginate and MSCs (139). This capsule composition improved the deleterious effect of encapsulation on human islets *in vitro*, showing decreased caspase activity and increased VEGF secretion. Improved islet outcome was possibly related to the cytoprotective function of MSCs whose paracrine effect was enhanced by the presence of RGD motif (140, 141). Therefore, MSCs and RGD G-rich alginate capsules could substantially ameliorate survival and function of encapsulated human islets *in vitro* although further studies are still needed to validate these results *in vivo*.

To date, it remains obscure whether pericapsular fibrosis overgrowth (PFO) occurring in an allograft environment could

be alleviated after co-inclusion of MSCs and islet. PFO mainly involves macrophages and fibroblasts, which is related to the low survival rate of encapsulated islets (142). Therefore, islet co-encapsulation with MSCs was investigated as a strategy against PFO. It was previously validated that tumor necrosis factor-α (TNF-α) or interferon-γ (IFN-γ) can induce immunosuppressive activity of MSCs by producing cyclooxygenase-2 (Cox-2) and PGE2 before transplantation (143). Thus, Vaithilingam et al. used the co-encapsulation of islets with MSCs either unstimulated or stimulated by a mixture of cytokines IFN-γ and TNF-α to test PFO and islet survival. C57BL/6 mice were used for the transplantation with strong immune response and fibrotic response that were similar to that in humans. The results indicated that a slight decrease of PFO in the stimulated mice was sufficient to significantly improve graft survival and islet activity (144). Immunosuppression in the stimulated MSC group was correlated to increased production of NO, which played a major role in regulating T cell immune response (145, 146). However, prior to clinical translation for patients with T1DM, further studies should be conducted using stimulated human bone-derived MSCs (BMSCs) co-encapsulated together with human islets in allograft settings using humanized mouse models.

PANCREATIC PROGENITOR CELL AS ANOTHER RESOURCE OF β-CELLS

Progenitor cells are considered as cells which have the ability to differentiate into a specific target cell. Studies have shown that β-cell progenitor cells derived from human embryonic stem cells express high levels of NKX6-1 and are prone to further mature into glucose-responsive β-cells (76). The key difference between progenitor cells and stem cells is that stem cells have an unlimited proliferation capacity, whereas progenitor cells can divide only a limited number of times (147). There are several theories about the origin of pancreatic progenitor cells. Mostly accepted is that islet progenitor cells are derived from pancreatic ducts, where they regenerate, differentiate and migrate to form new islets (148, 149). Studies have shown that islet formation starts early in embryonic development after birth, through the migration of pancreatic primordial cells out of epithelial ducts to form clusters of epithelial cells. Later-on these cells differentiate then into hormone-producing endocrine cells (150, 151). Formation of new islets, meaning the differentiation of islet progenitor cells into new islets, in or near ducts, has long been considered as an active process occurring after birth (152, 153). In a genealogy-tracing experiment to genetically label duct cells, the Cre-Lox system, in which Cre recombinase expression was driven by the promoter of carbonic anhydrase II (CAII), a marker of mature ducts cells, was used. Thus, pancreatic duct cells expressing CAII, have been shown to generate new islets as well as acini after birth and injury (154, 155). In addition, another potential source for β-cells is the islet itself. *Ex vivo* proliferation of β-cells or the plasticity of α-cells are still interesting concepts for generating β-cells (156). In conclusion, islet progenitor cells may exist not only in ductal epithelium, but also in islets itself (148, 157).

Several *in vitro* studies have shown that insulin-producing cells can be differentiated from adult pancreatic duct tissues (158, 159). Bonner-Weir et al. cultured adult duct tissue with matrix gel and observed islet buds composed of cytokeratin 19 (CK19) positive duct cells and insulin-positive cells (72). Other studies have also shown that some CK19-positive ductal epithelial cells differentiate into endocrine cells (160). Also, progenitor-like cells isolated from the adult pancreas formed tubular mature annular/dense colonies expressing PDX-1 and SOX9 and differentiated into endocrine/acinar colonies. Most endocrine/acinar colonies contained a majority of β-like cells which expressed and secreted insulin and C-peptide (161, 162). Further, duct cells under the action of glucagon-like peptide-1 (GLP-1) differentiate into islet endocrine cells including β-cells *in vitro* (163, 164). In addition, it has been early substantiated that β-cells are regenerated by duct cell trans-differentiation. Islet and pancreatic regeneration are achieved by replication of β-cells near or inside the pancreatic ducts, or by progenitor cells expressing Ngn-3 (165, 166).

Interestingly, progenitor cells from outside of the pancreas, such as murine skeletal muscle-derived progenitor cells, have been differentiated into insulin-producing clusters by a differentiation protocol comprising four steps of culture. These progenitor cells transformed into mature β-cells during development and significantly reduced hyperglycemia and improved survival after transplantation into STZ-induced diabetic mice (167). Moreover, Yi Arial Zeng's team identified a new population of protein C receptor-positive (Procr+) endocrine progenitor cells by single-cell RNA sequencing and that did not express known endocrine or exocrine differentiation markers of the adult mouse pancreas (168). In clonal density culture, islets can be formed stably, thus exerting its hypoglycemic function *in vivo*. It was also found that transplantation of pancreatic progenitor cells under the mammary fat pad or renal capsule did not affect their eventual differentiation into functional β-cells, despite no exposure to the "pancreatic" microenvironment (169). In conclusion, pancreatic progenitor cells capable of forming islet-like structures *in vivo* derived from human pluripotent stem cells represent a potential cell source for the treatment of type I diabetes.

DISCUSSION AND SUMMARY

Worldwide, it is estimated that there are currently 463 million persons with diabetes and this number is projected to reach 578 million by 2030, and 700 million by 2045. About 10 percent of those have T1DM, while type 2 diabetes mellitus (T2DM) is the most common type of diabetes that accounts for about 90 percent of all diabetes cases. With a variety of common and predisposing complications, 10% of global health expenditure (USD 760 billion) is spent on the prevention and cure of diabetes.

In this review, we expose recent advances in the development and use of pancreatic progenitor cells, bone marrow stem cells (MSCs), embryonic stem cells (ESCs) and pluripotent stem cells (PSCs) as cell sources for engineering islets for future β-cell replacement therapies, with a focus on recent biotechnology engineering. Although a significant progress has been achieved for the development of islet clusters from human stem cells, only a few

practical applications of bioengineering technology are realized in the treatment of diabetes. For the treatment of T1DM, novel attempts have focused on the development of bioengineering strategies such as microencapsulation with the aim to avoid immunosuppressive agents (170). For the treatment of T2DM, the exploration for new treatments includes stem cell differentiation, drug therapy and other methods (171). Although autologous iPSCs could theoretically be optimal for clinical use by avoiding immune rejection, long-term results of iPSC-differentiated cells transplantation still needs further confirmation.

Besides considerable advances made in the field, such as improved protocols for endocrine differentiation from SC, validation of therapeutic efficiency within animals and humans remains limited. Another challenge in this field is the lack of allogeneic and autoimmune humanized T1DM models to study the efficacy and safety of various stem cell therapy or devices. Given the major challenge remaining for the clinical applications, the hurdles include immune rejection, recurrence of autoimmunity, genetic stability and risk of tumorigenesis. Genetic instability could aggravate the risk of tumorigenicity, while *de novo* mutations in mtDNA obtained from reprogramming to the iPSCs stage, long-term culture and differentiation into target cells and the immune environment of the transplantation site could activate immune response in autologous transplantation. However, evidence on the efficacy of immunosuppressive molecules in suppressing allogeneic immune responses remains obscure. Programmed death ligand-1 (PD-L1), a member of the CD28 T cell family, binds to programmed cell death (PD-1), and this could downregulate T cell proliferation and inhibit immune responses, which hypothetically prevents allograft rejection in organ transplantation (172). PD-L1 knockout caused the acceleration of cardiac allograft rejection in animal models, while clinical data from endomyocardial transplant biopsies and explant hearts indicated that the relative reduction of PD-L1 expression compared with PD-1 could be a defining pathologic feature (173). Cytotoxic T lymphocyte antigen 4-immunoglobulin fusion protein (CTLA4-Ig) blocks T cell co-stimulatory pathways while PD-L1 activates T cell inhibitory pathway, thus they function jointly in maintaining peripheral tolerance by suppressing T cell activity. The joint knock-in of CTLA4-Ig and PD-L1 in human embryonic stem cells (hESCs) successfully led to the immunoprotection of hESCs-derived teratomas, fibroblasts, and cardiomyocytes in humanized mice (Hu-mice) (174). Besides, PD-1/PD-L1 checkpoint axis is substantiated with its predominant role in regulating immune response in human heart transplant recipients and a mouse model of heart transplant rejection. Reduced graft endothelial PD-L1 expression was negatively relevant to the proportion of CD8 +T-cell infiltration in human heart transplantation, meanwhile, the abrogation of graft endothelial PD-L1 expression may facilitate acute rejection and lead to decreased graft survival (175). Therefore, the protective strategies addressing immune response without requiring systemic immune suppression are urgent to be developed. Herein, systemic use of exogenous PD-L1-Ig, overexpression of PD-L1 in transplanted cells and tissue overexpression of PD-L1 before transplantation are three promising strategies in preclinical induction of immune tolerance with PD-1 signaling (176). Furthermore, clinical data indicated the considerable relevance between PD-L1 expression in HSCs and degree of T cell apoptosis, which conferred further research potential in allogeneic transplantation of HSCs (177).

Future countermeasures and therapy strategies may benefit from a better knowledge of molecular pathways that affect immune conditions, such as the PD1/PD-L1 checkpoint axis, contribute to reducing graft rejection risk of patients, and effectively promote the development of stem cell-derived therapies. Also, ongoing research is needed to stimulate bioengineering technologies toward long-term functional medical devises for the radical cure of diabetes, and benefiting diabetic patients.

AUTHOR CONTRIBUTIONS

LB and YW conceived and designed the review. SD and YL wrote the manuscript. QZ prepared the figures. LB, CG-G and YW reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Single-Cell Landscape of Mouse Islet Allograft and Syngeneic Graft

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Islet transplantation to treat the late stage of type 1 diabetic patient (T1DM) has recently made inspiring success in clinical trials. However, most patients experience a decline in islet graft function in one to three years due to immune rejection. Although the mechanisms of immune cells, including macrophages, dendritic cells (DCs), neutrophils, natural killer cells (NKs), B cells, and T cells, that mediate immune rejection have been investigated, the overall characteristics of immune infiltrates in islet allografts and syngeneic grafts remain unclear. Single-cell RNA sequencing (scRNA-seq) has provided us with new opportunities to study the complexity of the immune microenvironment in islet transplants. In the present study, we used scRNA-seq to comprehensively analyze the immune heterogeneity in the mouse model of islet transplantation. Our data revealed T lymphocytes and myeloid cells as the main immune components of grafts 7 days post-islet transplantation, especially in allografts. Moreover, our results indicated that allogeneic islet cells were transformed into antigen-presenting cell-like cells with highly expressed MHC class I molecules and genes involved in MHC class I-mediated antigen presentation. This transformation may dramatically facilitate the interaction with cytotoxic $CD8^+$ T cells and promote the destruction of islet allografts. Our study provides insight into the transcriptomics and diverse microenvironment of islet grafts and their impacts on immune rejection.

Keywords: the immune atlas, allograft, single-cell RNA sequencing, immune heterogeneity, islet, Beta cell, islet transplantation, diabetes

Abbreviations: scRNA-seq, single-cell RNA sequencing; T1DM, type 1 diabetes mellitus; APC, antigen-presenting cells; VEC, vascular endothelial cell; Mes, mesenchymal cell; Tconv, conventional T cell; DC, dendritic cell; NK, natural killer cell; NKT, natural killer T cell; MΦ, macrophages.

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INTRODUCTION

Type 1 diabetes mellitus (T1DM) is caused by multiple factors, such as genetic and environmental factors, that lead to the autoimmune destruction of β cells (1–3). For late-stage T1DM patients, especially those with brittle diabetes, it is difficult to control various complications, such as cardiovascular disease, retinopathy, nephropathy, and life-threatening asymptomatic hypoglycemic coma, with exogenous insulin administration (4). Islet transplantation, when successful, can achieve so (5, 6). With the use of effective immunosuppressive agents, although most patients achieved insulin independence within the first year, they deteriorated as the islet graft declined rapidly afterward (7).

After islet allotransplantation, once the immune system is activated, macrophages, dendritic cells (DCs), neutrophils, natural killer cells (NKs), B cells, and T cells migrate into the graft, drive the proinflammatory cascade and destroy the graft (8, 9). As a result, the therapeutic efficacy of islet transplantation has also been largely limited by immune rejection.

Antigens of donor islet grafts, such as insulin, insulinoma-associated protein-2, glutamate decarboxylase, and zinc transporter 8, activate DCs and macrophages, which subsequently activate T cells and B cells (9). These antigens are recognized by the host immune system through the direct or indirect presentation. The direct presentation involves the immediate recognition of islet graft-derived antigen-presenting cells (APCs) and activation of host T cells. The indirect presentation involves the presentation of antigens of the graft by host APCs, thereby activating the host immune system.

After islet transplantation, $CD4^+$ T cells activate the $CD8^+$ T cell response, and M1 macrophages polarize and stimulate antibody production by B cells. A previous study proved that islet graft survival was prolonged when transplanted in $CD4^+$ T cell knockout mice with decreased $CD8^+$ T cell activity. Thus, $CD8^+$ T cell-mediated immune responses play an important part in islet rejection (10–12). $CD8^+$ T cells destroy islet graft cells by granule release-mediated cytolytic activities through activating Fas pathways (13, 14) and the production of IFN-γ (15). The specific attack of islet grafts by alloreactive $CD8^+$ T cells also constitutes a major component of islet allograft rejection, especially by the $CD103^+$ $CD8^+$ T cell subpopulation (16).

The role of NKs in islet allotransplantation remains controversial. Several studies have demonstrated that liver NKs contribute to islet destruction after intraportal transplantation (17–19). In contrast, other studies proved that NKs promote islet transplantation tolerance by a perforin-dependent mechanism or by B cell-dependent tolerance (20, 21).

M1 macrophage polarization is one of the main factors contributing to the proinflammatory environment of islet grafts, which can lead to reduced graft function (22). On the other hand, M2 macrophages are anti-inflammatory (23, 24). By inhibiting the activation of M1 macrophages or promoting the activation of M2-type macrophages, the survival of islets is prolonged (22–26).

To date, the overall immune characteristics within islet grafts remain unclear. The development of single-cell RNA sequencing (scRNA-seq) has provided us with new opportunities to study the molecular characteristics of the immune microenvironment in islet transplants at the single-cell level. Moreover, scRNA-seq can identify potential cell-cell interactions by profiling receptor-ligand transcriptomics of individual cells (27, 28). To date, in the field of islet transplantation, there are no reports of immune atlases in islet grafts at single-cell resolution. Here, we used scRNA-seq to comprehensively analyze the immune heterogeneity in islet grafts and compared the transcriptome variances between syngeneic islet transplantation and allografts.

MATERIALS AND METHODS

Animals

Wild-type 6-8 weeks old male C57BL/6 and BALB/c mice were purchased from Guangdong Medical Laboratory Animal Centre. All mice were maintained in specific pathogen-free conditions at the Central Laboratory of Shenzhen Longhua District Central Hospital. C57BL/6 mice were given a single intraperitoneal dose of 250 mg/kg body weight STZ (Sigma-Aldrich, St Louis, MO) in 0.5 M sodium citrate buffer. Two days after STZ administration, blood glucose levels were measured every day at 9:00-10:00 AM, mice with blood glucose levels steady above 16.8 mmol/L for 5 consecutive days were defined as diabetes. The animal protocols were approved by the Institutional Biomedical Research Ethics Committee of Guangdong Medical University.

Islet Isolation and Purification

Islets were isolated as previously described (29). Briefly, we first perfused the pancreas *in situ* with 1 mg/mL Collagenase Type V (Sigma-Aldrich) *via* the common bile duct. The inflated pancreas was dissected and further digested with an additional 1 mg/mL Collagenase Type V in a 37°C water bath for 15 min. After brief vertexing, the islets were purified by discontinuous gradient centrifugation. Isolated islets were maintained in the complete CMRL-1066 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) for grafting.

Islet Transplantation

For the syngeneic islet transplantation, 500 donor islets (C57BL/6 origin) were transplanted under the kidney capsule of one C57BL/6 recipient mice (n=5). For the allogeneic islet transplantation, 500 donor islets (BALB/c origin) were transplanted under the kidney capsule of one C57BL/6 recipient mice (n=5), as the allogeneic graft. A small incision was made at the pole of the kidney capsule. The islets were injected under the capsule through the incision using a pipette tip. The incision in the body wall was sutured.

Graft Harvest and Dissociation of Single Cells

Seven days after transplantation, the mice were sacrificed, and the grafts adhering to the kidney capsule were harvested. The grafts were disintegrated with 0.01% (w/v) Liberase TH and 100 U/mL DNase I in RPMI 1640 for 10 min. Cells were then

filtered through a 40-μm cell strainer and washed with 5 mL washing buffer (1 X PBS with 2 mM EDTA and 0.5% BSA), followed by centrifugation at 200 g for 5 min. After centrifugation, the cells were then suspended at a concentration of 1 X 10^6 cells/mL in RPMI 1640-10% FBS and held on ice.

Single-Cell Library Construction and Sequencing

After dissociation, the concentration of single-cell suspension was adjusted to 700–1,200 cells/μL. Cell viability was determined by trypan blue staining with a TC20 automated cell counter (Bio-rad, Hercules, CA). The ratio of viable cells was required to be more than 85%. The input cells were then loaded onto the channel of a Single Cell B Chip (v3 chemistry, PN-1000153) and loaded onto a Chromium Controller (10x Genomics, Pleasanton, CA) to generate single-cell GEMs (gel beads in the emulsion). Reverse transcription and library preparation were performed using the 1 Chromium Single Cell 3' Reagent Kits following the 10x Genomics protocol. Libraries were sequenced, aiming at a minimum coverage of 50,000 raw reads per cell on an Illumina NovaSeq 6000 by Novogene Bioinformatics Technology Co., Ltd. (Tianjin, China).

ScRNA-Seq Data Analysis

For syngeneic and allogeneic graft collected by our group: Sequences obtained from sequencing using the 10x Genomics single-cell RNA-sequencing platform were demultiplexed and mapped to the mm10 transcriptome using the Cell Ranger package (10x Genomics). Cells were removed if they expressed fewer than 200 unique genes, more than 4,500 unique genes, or greater than 15% mitochondrial reads. Genes not detected in at least 3 cells were removed from subsequent analysis.

To analyze the single-cell RNA-seq data, we performed Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) and t-distributed Stochastic Neighbor Embedding (t-SNE) using the Seurat R Package (version 3.1.5) with the first 75 principal components after performing the principal component analysis (PCA) on the 2000 most variable genes. Identification of significant clusters was performed using the FindClusters algorithm in the Seurat package with the resolution set as 0.6. Marker genes for each significant cluster were found using the Seurat function FindAllMarkers. Cell types were determined using a combination of marker genes identified from the literature and the CellMarker web tool (http://biocc.hrbmu.edu.cn/CellMarker/).

For public scRNA data of GSE84133-GSM2230761 and GSE84133-GSM2230762: We obtained two single-cell RNA-seq datasets of mouse pancreas (GSE84133-GSM2230761 for islets of 5 ICR mice; GSE84133-GSM2230762 for islets of 5 C57BL/6 mice) (30).

Basic filtering, classification, and visualization of the mouse pancreas dataset were performed by the Seurat R package (v3.1.5). Cells expressing fewer than 200 or more than 4,500 unique genes were filtered. The top 1000 variable genes were used for further analysis. The function FindMarkers was used based on the t-test. Ten principal components (PCs) remained for uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) analysis. Cell types were identified by canonical markers.

Cell-Cell Communication Analysis

CellPhoneDB was used to infer enriched ligand-receptor interactions among different cell types (27). Count data were used as input for the CellphoneDB algorithm (version 2.1.2) in Python 3.6. Ligand–receptor pairs are defined based on physical protein-protein interactions from the information in the CellPhoneDB website (www.CellPhoneDB.org). To identify the most relevant interactions between cell types, we looked for the cell type-specific interactions between ligands and receptors. Only interaction pairs with a p-value < 0.05 remained for the heatmap plot generated by CellPhoneDB. Chemokines, costimulatory molecules, and coinhibitory molecules were selected for visualization of interaction pairs between each two cell types. The results were visualized using the dot_plot function of CellphoneDB.

Immunofluorescent Staining

Islet graft with the host kidney was fixed with paraformaldehyde, paraffin-embedded, and sectioned. For immunofluorescent staining, briefly, incubate the sections in two changes of xylene, dehydrate the sections with gradient ethanol, retrieve antigen in EDTA antigen retrieval buffer (pH 8.0), block endogenous peroxidase in 3% H_2O_2 for 25 min, block with 3% BSA at room temperature for 30 min, incubate slides with the first primary antibody overnight at 4°C in a wet box, incubate slides with the secondary antibody (respond to first primary antibody in species) at room temperature for 50 min in dark, incubate slides with TSA-FITC solution for 10 min in dark, remove the unbound primary antibodies and secondary antibodies with antigen retrieval procedure, incubate slides with the second primary antibody overnight at 4°C in a wet box, incubate slides with the CY3-labeled secondary antibody (respond to second primary antibody in species) at room temperature for 50 min in dark, quench spontaneous fluorescence for 5 min, incubate the slides with DAPI solution at room temperature for 10 min in dark, mount the slides with anti-fade mounting medium. Images were captured by Pannoramic MIDI (Hungary, 3DHISTECH).

Statistical Analysis

For the analysis of gene expression in scRNA-seq data, all single-cell sequencing data statistical analyses were performed in the R Seurat package (3.1.5). The Wilcoxon rank-sum test was applied for comparisons in two groups. Statistical significance was accepted for $p < 0.05$.

For differentially expressed genes analyzed by the limma package in R (4.0.5), genes with a cutoff of p-value < 0.05 and fold change > 1.2 were determined to be differentially expressed. Heatmaps were generated by the row-scaled expression values using the pheatmap package in R (4.0.5).

RESULTS

Single-Cell Analysis Reveals Dramatic Changes in Cell Heterogeneity Between Islet Allografts and Syngeneic Grafts

The pretransplant blood glucose levels of transplant recipients (STZ induced diabetic mice) were higher than 20 mmol/L **(Supplementary Figure 1)**. Both syngeneic islet transplantation (C57BL/6 islets to C57BL/6 recipients) and allogeneic islet transplantation (BALB/c islets to C57BL/6 recipients) restored the blood glucose of STZ mice to normal levels from 1 to 7 days after transplantation **(Supplementary Figure 1A)**. However, immunofluorescence staining of INSULIN$^+$ islet cells revealed profound destruction of islet cells in allogeneic transplantation in P14D **(Supplementary Figure 1B)**. We performed single-cell RNA sequencing (scRNA-seq) on islet allografts (BALB/c islets to C57BL/6 recipients) and syngeneic grafts (C57BL/6 islets to C57BL/6 recipients) to comprehensively identify the cell components and variations that are related to graft rejection **(Figure 1A)**. Seven days after transplantation, the grafts were harvested and subjected to 10x Genomics pipeline barcoding, library preparation, and sequencing **(Figure 1A)**. After computational quality filtering, the transcriptomes of 19,640 single cells, including 11,870 cells from allografts and 7,770 cells from syngeneic grafts, were obtained.

Using a graph-based clustering approach and the uniform manifold approximation and projection (UMAP) dimensionality reduction method in the Seurat package (31), we identified major populations of graft-infiltrated immune cells and islet cells based on their feature gene expression, including macrophages (MΦs), conventional T cells (Tconvs), B cells, CD8$^+$ T cells, dendritic cells (DCs), vascular endothelial cells (VECs), islet cells, mesenchymal cells (Mes), natural killer cells (NK), and regulatory T cells (Tregs) **(Figures 1B, C**; **Supplementary Table 1)**. The major immune cell populations were composed of T cells, including CD4$^+$ T cells (marker: *Cd4*), CD8$^+$ T cells (marker: *Cd8a*), macrophages (MΦ, marker: *Cd68*, *Fcgr3a*), dendritic cells (DCs, markers: *Clec9a*, *Flt3*), natural killer cells (NKs, markers: *Ncr1*, *Klra9*) and a small population of B lymphocytes (markers: *Cd19*, *Ms4a1*) **(Figure 1D)**. We also captured several nonimmune cell types, including mesenchymal cells (markers: *Col3a1, Fbn1*), vascular endothelial cells (markers: *Pecam1, Cdh5*), and islet cells (markers: *Ins, Chga*) **(Figure 1E)**.

Compared with syngeneic grafts, most of the immune infiltrates in allografts were represented by T cells, macrophages, DCs, NKs, and B cells **(Figures 1F, G)**. Specifically, T cells represented the most abundant immune infiltrates in islet allografts, including CD4$^+$ Tconv cells, Tregs, and CD8$^+$ T cells **(Figure 1G)**. Macrophages then represented the second most abundant immune infiltrates in allografts, while NKs, DCs, and B cells were very limited in number **(Figures 1F, G)**. In contrast to immune cells that were more enriched in islet allografts, mesenchymal cells and vascular endothelial cells accumulated with the maintenance of islet cells in syngeneic grafts **(Figures 1F, G)**. To exclude the possibility that infiltrating immune cells were donor-derived tissue-resident cells, we analyzed the scRNA-seq data of healthy C57BL/6 and ICR mouse islet cells from a public database (30). These data indicated that islet-resident immune cells in baseline islet were very limited, and only a small population of macrophages was identified **(Supplementary Figures 2A–D)**. The low portion of immune cells was consistent with a previous study of human healthy pancreatic islets (32).

T Cells Were Recruited and Activated in Islet Allograft

To reveal the functional characteristics of T cells in allografts, we performed unsupervised clustering of all T cells defined in our initial analyses **(Figure 1B)**. A total of five subtypes were identified, including one cluster of cells showing high proliferating potential, one cluster of CD8$^+$ T cells, one cluster of natural killer T cells, and two clusters of CD4$^+$ T cells **(Figures 2A, B)**. All T cell subtypes were much more enriched in allografts than in syngeneic grafts **(Figure 2C).**

T1 cells expressed high levels of genes associated with cell division, including *Stmn1*, *Top2a*, and epigenetic regulators *Dnmt1, Ezh2* **(Figure 2D)**. These indicated that T1 cells were constantly dividing and proliferating, and that epigenetic regulation was possibly required for T cell function in acute rejection (33–35). CD8$^+$ T2 cells as well as CD8$^+$ T1 cells expressed natural killer cell inhibitory receptors *Klrc1, Klrd1*, and natural killer cell granule protein *Nkg7*, granular enzyme gene *Gzmk* **(Figure 2E)**, these indicated that they were activated cytotoxic cells (36, 37). It is worth noting that these cytotoxic CD8$^+$ T cells, especially those from allograft, highly expressed *Xcl1 and Ccl5* **(Figure 2F)**, which are chemoattractants for blood monocytes (38, 39). CD8$^+$ T1 and T2 cells from allografts also increased expression of transcription factor *Hif1a*, dehydrogenase *L*dha, and tumor necrosis factor receptor superfamily member *Tnfrsf4* **(Figure 2F)**. These genes were responsible for the proliferation and cytotoxicity of T cells (40, 41).

T3 cells did not express both *Cd4* and *Cd8* **(Figure 2B)**, but expressed *Klra1, Klrb1C, Cd122,* and *Cd7*. **(Figure 2G)**, indicating that they represented a population of CD7$^+$ CD122$^+$ natural killer T cells (NKT) (42, 43). They also showed an activated cytotoxic phenotype as they expressed *Klrc1,Klrd1, Nkg7,* and *Gzmk* **(Figure 2E)**. CD4$^+$ T cells (T4 and T5) were composed of CD4$^+$ conventional T cells (Tconv, *Tcf7*, *Tnfsf8*) **(Figure 2H)** and Treg cells (*Il2ra*, *Foxp3*) **(Figure 2I)**.

To further study the molecular variations in T cells between syngeneic grafts and allografts, we analyzed their transcriptome differences. Allograft induced dramatic gene expression changes in infiltrating CD8$^+$ cytotoxic T cells **(Figure S3A)**. Gene ontology (GO) pathway enrichment analysis revealed immune response and cell-cell adhesion as the top enriched signatures that differed in syngeneic grafts and allografts **(Figure S3B**; **Table S2)**. Comparison of feature gene expression between the syngeneic and allogeneic groups further identified *Ccr7*, *Cxcr3*, and *Cxcr4*, which were upregulated in allograft infiltrating CD8$^+$ T cells **(Figure S3C)**. The chemokine receptor *Ccr7* is required for T cell activation in inflammation and infection (44). *Cxcr3*

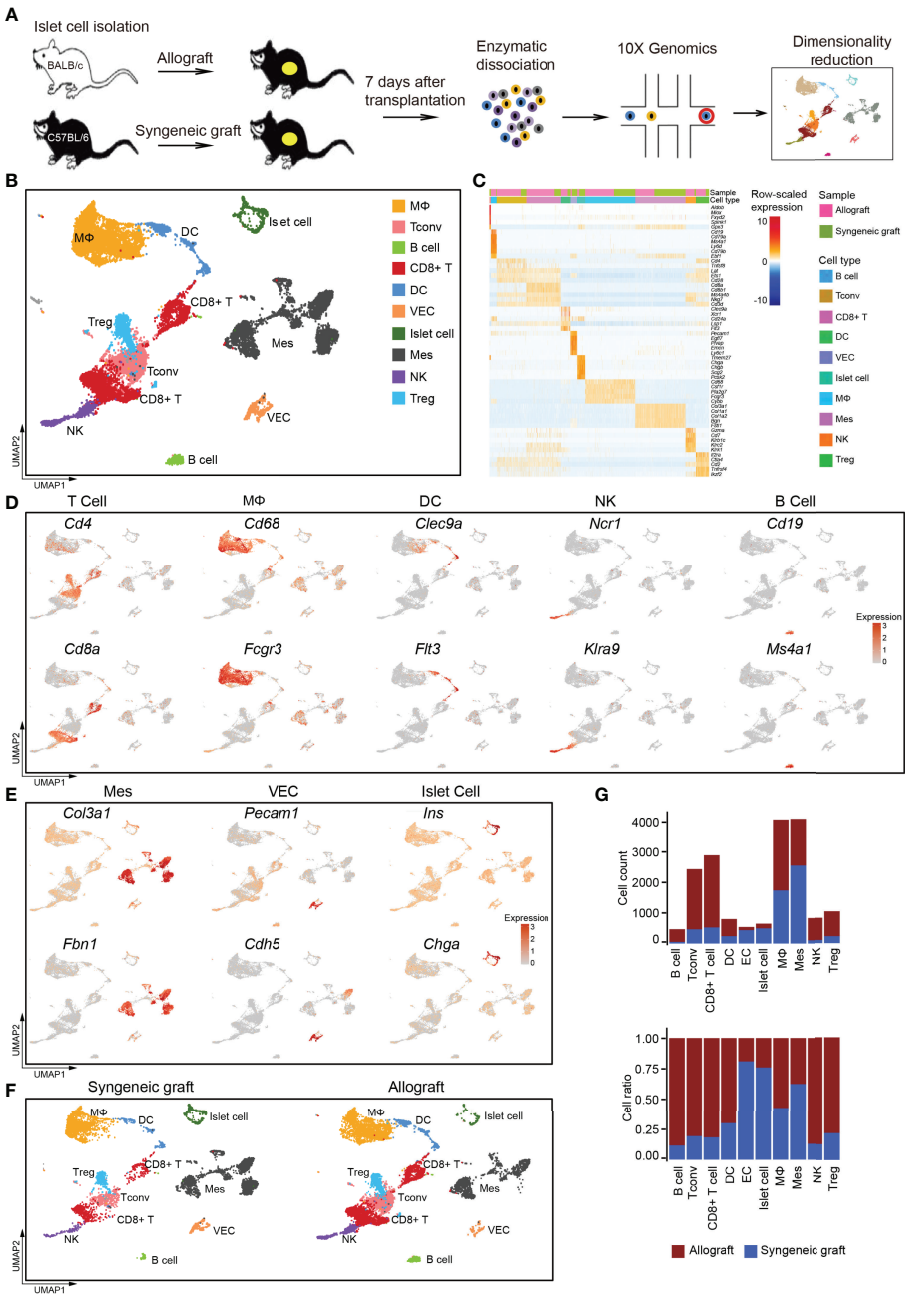

FIGURE 1 | Overview of the components of cells in islet cell grafts 7 days post-transplantation by single-cell RNA-seq. **(A)** Schematic of the experimental design, single-cell sequencing, and analysis. **(B)** UMAP visualization of the total cells profiled here, with each cell colorcoded for the associated cell type. **(C)** Heatmap of row-scaled expression of marker gene expression within defined populations. Expression was measured in units of log2. **(D)** UMAP visualization shows the expression of marker genes for T cells, macrophages, dendritic cells (DCs), natural killer cells (NKs), and B cells. **(E)** UMAP visualization shows the expression of marker genes for mesenchymal cells, vascular endothelial cells, and islet cells. **(F)** UMAP visualization of the total cells from the syngeneic graft (left) and allograft (right), with each cell colorcoded for the associated cell type. **(G)** The number (upper panel) and a fraction (lower panel) of cells in the indicated cell type. Syngeneic graft n = 5, allograft n = 5. Analysis of gene expression in scRNA-seq data was performed in R using Seurat. VEC, vascular endothelial cell; Mes, mesenchymal cell; Tconv, conventional T cell.

and *Cxcr4* are chemokine receptors that are highly expressed on effector T cells and play important roles in T cell trafficking and function (45, 46). Thus, the abundance of activated T cells in allografts indicates their function in graft rejection, and the selective inhibition of these chemokine receptors, such as *Cxcr4*, may protect the graft from immune attack (46). Consistent with the abundance of T cells from allografts in our scRNA-seq data, immunofluorescence staining of graft sections

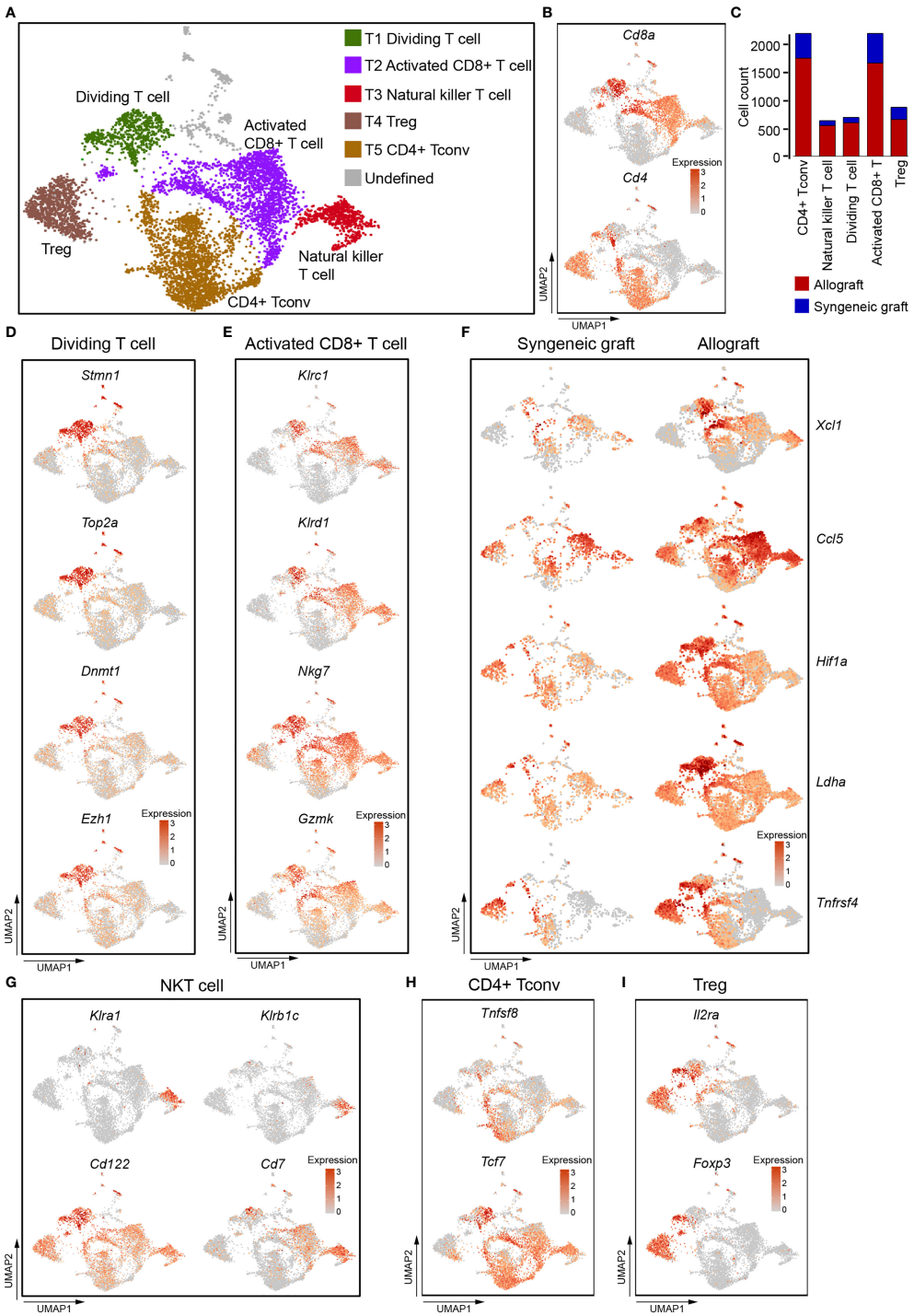

FIGURE 2 | Single-cell data revealed molecular details and subclusters of T cells. **(A)** UMAP visualization of the total T cells, with each cell colorcoded for the associated cell subtype. **(B)** UMAP visualization of the distribution of $CD4^+$ T cells and $CD8^+$ T cells by the expression of *Cd4* and *Cd8a*. **(C)** The number of cells in the indicated cell type. **(D)** UMAP visualization shows the expression of *Stmn1, Top2A, Dnmt1, Ezh2* for dividing/proliferating T cells. **(E)** UMAP visualization shows the expression of marker genes for activated $CD8^+$ T cells. **(F)** UMAP visualization shows the increased expression of *Xcl1, Ccl5, Hif1A, Ldha*, and *Tnfrsf4* in activated $CD8^+$ T cells from allografts. **(G–I)** UMAP visualization of the expression of curated feature genes specific for NKT cells **(G)**, CD4 Tconv **(H)**, and Treg **(I)**. Tconv, conventional T cell; Treg, regulatory T cell.

showed much more $CD4^+$ T cells **(Figure 3A)** and $CD8^+$ T cells **(Figure 3B)** in allograft compared with syngeneic graft.

Macrophages Are the Main Antigen-Presenting Cells in Ectopic Islet Allograft

The scRNA-seq analysis of 4,286 myeloid cells that highly expressed *Cd68*, *Cd32*, and *H2-Aa* revealed six transcriptionally distinct clusters, including three clusters of macrophages (MΦ-C1, MΦ-C2, and MΦ-C3) and three clusters of DCs (DC-C4, DC-C5, and DC-C6) **(Figures 4A**; **Supplementary Figure 4A, B)**. Among these, macrophages represented the most abundant myeloid cell type. Except for cluster MΦ-C1, which contained cells mainly from syngeneic grafts, all other clusters were more frequently enriched (>60%) in allografts **(Figures 4B, C)**. Macrophages were characterized by specific expression of functional markers, including *Cd68* and *Fcgr3a* **(Figure 1D)** and *Cd14*, *Adgre1* (F4/80), and *Mertk* **(Figure 4D)**. DCs specifically expressed major histocompatibility complex class II molecules (*H2-Aa*, *H2-Dmb2*) and the cell surface tyrosine-protein kinase receptor *Flt3* **(Figure 4E)**.

MΦ-C1 was mostly enriched from syngeneic grafts **(Figure 4C)**. It was characterized by elevated expression of *Fcrls*, *Igf1*, and *Stab1* **(Figure 4F)**. *Fcrls*, which was previously found to be expressed by central nervous system-associated macrophages (47), was also highly expressed in islet graft-infiltrated MΦ subsets. M2-like macrophages secrete IGF1, which in turn regulates their activation in response to immunometabolic challenges (37). In addition, we detected higher expression of other M2-associated genes, such as *Pf4*, *Ms4a7*, *Mrc1*, *Gdf15*, *Trem2*, and *Csf1r*, in the MΦ-C1 subset **(Supplementary Figure 4C)**.

MΦ-C2 macrophages were identified as inflammatory-activated macrophages, as they highly expressed the C-X-C motif chemokines *Cxcl9*, *Cxcl10*, complement *C3*, and other M1-like macrophage marker genes **(Figures 4G**, **Supplementary Figure 4D**; **Supplementary Table 3)**. This subset mainly consisted of cells from islet allografts **(Figure 4C).** A comparison between MΦ subsets revealed an activated phenotype for the MΦ-C2 cluster with higher levels of genes involved in antigen presentation and processing, chemokine production, cell surface proteins, and transcription factors. Of note was a high expression of CIITA and MHC molecules, including class I-related *H2-Q4* and *H2-T22* and class II-related *H2-aa*, *H2-Ab1*, *H2-Eb1*, and *H2-Dma* **(Figure 4H)**. Consistently, MΦ-C2 upregulated the expression of genes related to antigen processing, including *Tap1*, *Tap2*, *Cd74*, and proteasome subunits **(Figure 4H)**. In addition, MΦ-C2 exhibited canonical inflammatory features with high expression of several chemokines (*Cxcl9*, *Cxcl10*, *Cxcl16*, and *Il18bp*), cell surface receptors (*Itgax*, *Irgm1*, *Icam1*, *Klra2*, *Ly6a*, *Ly6c2*, and *Ly6i*), and activated IFNγ signaling (*Irf1*, *Irf2*, *Irf7*, and *Hif1a*) **(Figure 4H)** (48). The MΦ-C3 population showed some common gene expression features with MΦ-C1 and MΦ-C2 and had higher expression levels of *Mmp12*, *Lpl*, and *Gpc1* **(Supplementary Figures 4B, I)**.

We identified three subpopulations of DCs (DC-C4, DC-C5, and DC-C6), although with much lower abundance than macrophages **(Figures 4A–C)**. All DC subpopulations were enriched predominantly in allografts **(Figure 4C).** DC-C4

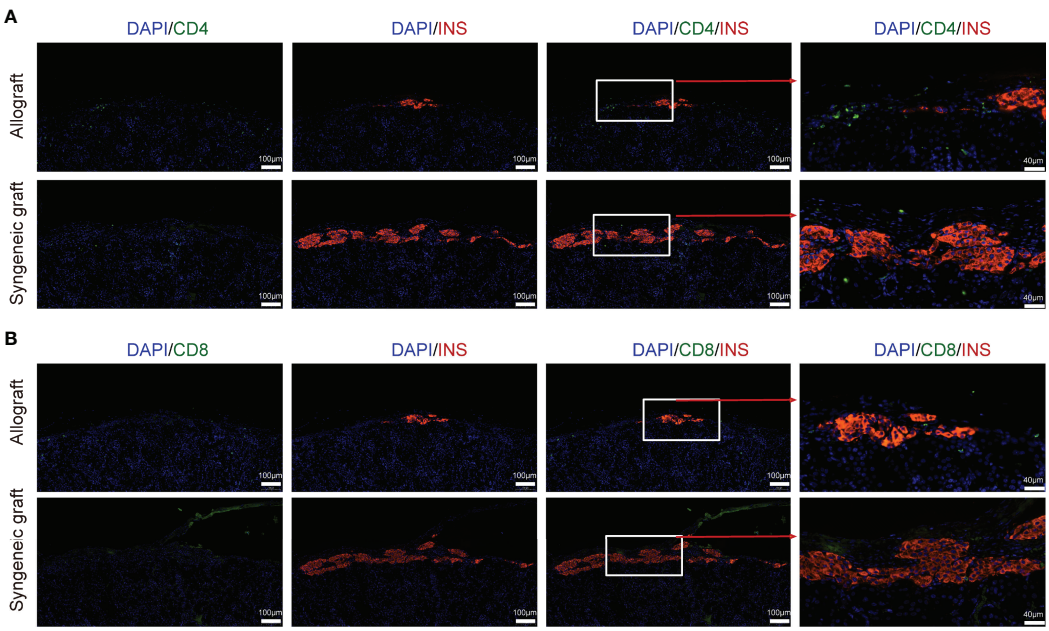

FIGURE 3 | Localization of T cells in islet allograft. **(A)** Representative image of a graft section stained with antibodies specific for Insulin (red), CD4 (green), and DAPI nuclear counterstain (blue). **(B)** Representative image of a graft section stained with antibodies specific for Insulin (red), CD8 (green), and DAPI nuclear counterstain (blue). Syngeneic islets graft (C57BL/6 islets to C57BL/6 recipients) and allogeneic islets graft (BALB/c islets to C57BL/6 recipients) were harvested 7 days post-transplantation and sectioned together with mouse kidney.

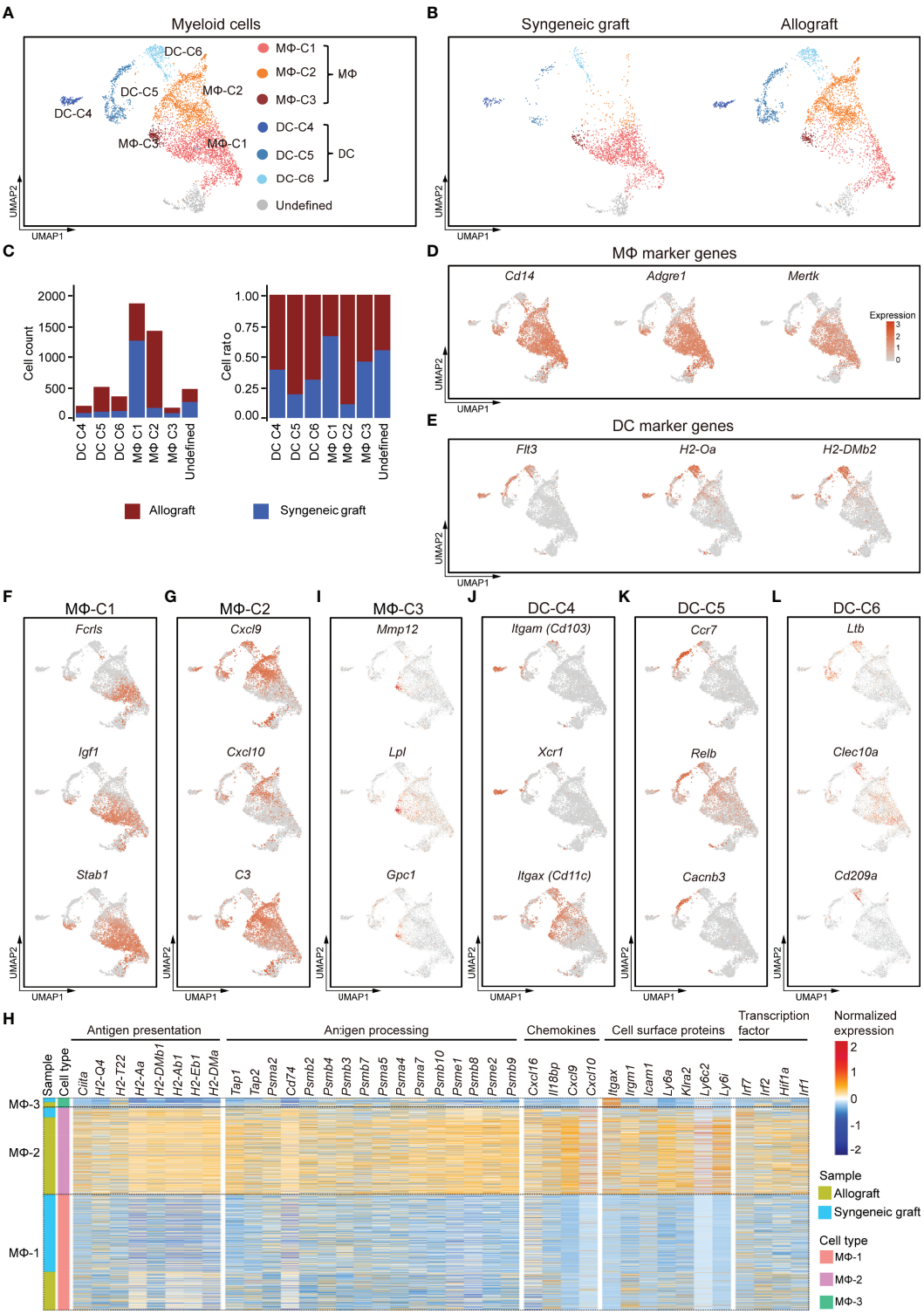

FIGURE 4 | Subclusters and molecular characteristics of myeloid cells infiltrated in islet grafts. **(A)** UMAP visualization of the total myeloid cells, with each cell colorcoded for the associated cell subtype. **(B)** UMAP visualization of myeloid cells from syngeneic grafts (left) and allografts (right), with each cell colorcoded for the associated cell type. **(C)** The number (left panel) and fraction (right panel) of cells in the indicated cell type. **(D, E)** UMAP visualization shows the expression of marker genes for macrophages **(D)** and DCs **(E)**. **(F–I)** UMAP visualization of the signature genes for 3 subclusters of macrophages: MΦ-C1 **(F)**, MΦ-C2 **(G)**, and MΦ-C3 **(I)**. **(H)** Heatmap showing row-scaled expression of representative genes within 3 subclusters of macrophages. Genes were categorized by their biological functions. Expression was measured in units of log2. **(J–L)** UMAP visualization of the signature genes for 3 subclusters of DCs: DC-C4 **(J)**, DC-C5 **(K)**, DC-C6 **(L)**.

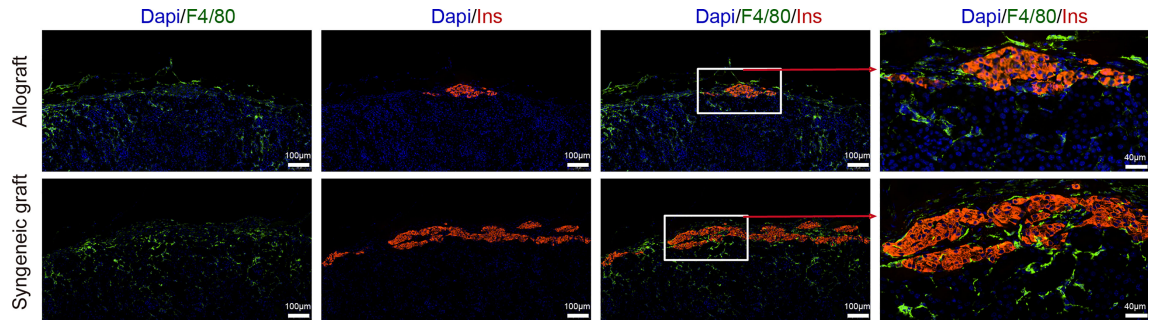

FIGURE 5 | Localization of macrophages in islet grafts. Representative image of a graft section stained with antibodies specific for Insulin (red), F4/80 (green), and DAPI nuclear counterstain (blue). Syngeneic islets graft (C57BL/6 islets to C57BL/6 recipients) and allogeneic islets graft (BALB/c islets to C57BL/6 recipients) were harvested 7 days post-transplantation and sectioned together with mouse kidney.

highly expressed *Itgae* (*Cd103*), *Xcr1*, and *Itgax* (*Cd11c*), which characterized this subpopulation as $CD103^+$ conventional DCs **(Figure 4J)** (49). The DC-C5 subpopulation had a $CD11B^-$ $CD11C^-$ phenotype **(Supplementary Figure 4E)** but highly expressed *Ccr7*, *Relb*, and *Cacnb3* **(Figure 4K),** indicating that this set represented a population of activated DCs (50, 51). The DC-C6 subpopulation was characterized by higher expression of *Ltβ*, *Clec10a*, and *Cd209a* **(Figure 4L)**. Taken together, inflammatory macrophages and DCs were enriched in islet allografts. Heterogeneity analysis revealed distinct responses of myeloid cells to xenogeneic stimuli.

In contrast to T cells, macrophages (including all 3 subclusters) showed similar abundance in allograft and syngeneic graft **(Figure 1G)**. Immunofluorescence staining of graft sections showed comparable macrophage infiltrates ($F4/80^+$) both in allograft and syngeneic graft **(Figure 5)**.

Islet Cells Were Activated to Facilitate $CD8^+$ T Cell Interactions in Ectopic Allografts

We then recovered three clusters of islet cells, most of which were obtained from syngeneic grafts, as expected because most of the islet cells in allografts were immune rejected **(Figures 6A–C)**. Based on the feature gene expression, these three clusters of islet cells were identified as β (Islet-C1), α (Islet-C2), ϵ (Islet-C3) cells **(Figures 6D–G)**. Islet-C1 was the most abundant cell subcluster and highly expressed β cell marker genes *Ins*, *Prlr*, and *Igrp* **(Figure 6E)**. Islet-C2 was a cluster of α cells that expressed marker genes *Gcg*, *Mafb*, and *Irx1* **(Figure 6F)**. Islet-C3 was identified as ϵ cells that highly expressed *Sst*, *Ly6h*, and *Ptprz1* **(Figure 6G)**.

Comparison of the gene expression features in islet cells from allografts with those from syngeneic grafts showed that IFN-inducible genes, including guanylate-binding protein (*Gbp2*, *Gbp4*, and *Gbp7*), transcription factor subunit *Stat1*, and interferon-inducible GTPase 1 (*Iigp1*, *Igtp*, and *Irgm1*), were upregulated in allograft islet cells **(Figure 6H)**. In line with the expression of IFN-inducible genes, receptors for interferon ligands, including *Ifngr1*, *Ifngr2*, *Ifnar1*, and *Ifnar2*, were also upregulated in allograft islet cells **(Figure 6I),** indicating that the islet cells in allografts were activated by interferons. Interestingly, we found that islet cells recovered from allografts highly expressed MHC class I molecules (*H2-K1*, *H2-D1*, *H2-T23*, *H2-Q4*, and *H2-Q7*) and genes involved in MHC class I-mediated antigen presentation (*Tap1*, *Tap2*, and *Tapbp*) **(Figure 6J).** In addition, islet cells from allografts also expressed a battery of proteasome subunit genes, such as *Psmb8*, *Psmb9*, *Psmb10*, *Psme1*, *Psme2*, and lysosome protease *Ctsl* **(Figure 6K)**. Together, these results indicated that islet cells in the xenogeneic microenvironment were potentially transformed into antigen-presenting cell-like cells. This transformation with high expression of MHC class I molecules specifically may facilitate the interaction of $CD8^+$ T cells.

Mesenchyme Stromal Cells Were Highly Heterogeneous in Islet Grafts

Mesenchymal cells establish a microenvironment for cell proliferation, differentiation, and immune intervention. As the transplanted islets originally contained mesenchymal cells that were recaptured in our 10x scRNA-seq dataset, we further reclustered the mesenchymal compartment. Based on the gene expression features, the mesenchymal cells were subdivided into nine transcriptionally distinct clusters **(Figure 7A)**. Among these, mesenchymal cluster 1 (Mes-C1) represented the specific enrichment of cells from syngeneic grafts **(Figure 7B)**. Mes-C1 highly expressed *Mest* **(Figure 7C)**, a negative regulator of Wnt signaling that was reported to affect neuronal differentiation (52, 53). In addition, Mes-C1 also highly expressed growth/differentiation factor 10 (*Gdf10*), transcription factor *Zim1*, and receptor tyrosine kinase *Epha4*, which may arm mesenchymal cells in an extracellular matrix organization **(Figure 7C)**. These results indicated that this subcluster of mesenchymal cells may support the reorganization of grafted islet cells. Mes-C2 was characterized by high expression of tissue factor *F3* and other extracellular signaling adaptors, including *Ltbp1*, *Smoc2*, and *Pi16*, indicating that these cells were active in cell migration

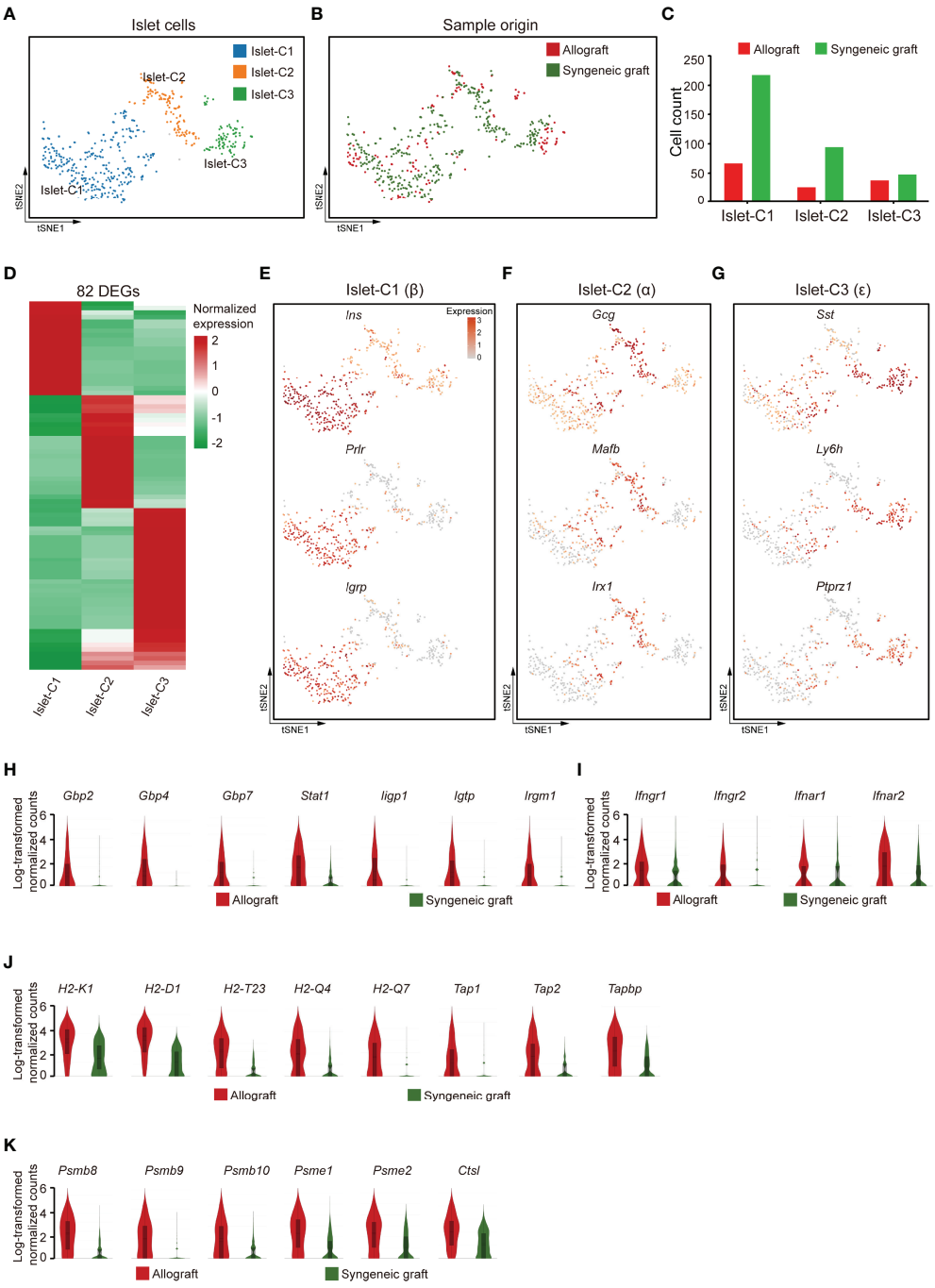

FIGURE 6 | Islet cells exhibited an activated gene expression signature 7 days post-allogeneic transplantation. **(A, B)** UMAP visualization of islet cells, with each cell colorcoded for the associated cell subtype **(A)** and sample origin **(B)**. **(C)** The number of islet cells in the indicated clusters. **(D)** Heatmap showing DEG expression within 3 subclusters of islet cells. **(E–G)** t-SNE visualization of the signature genes for 3 subclusters of islet cells: islet -C1 **(E)**, islet -C2 **(F)**, islet -C3 **(G)**. **(H–K)** Violin plots showing the smoothened expression distribution of IFN-inducible genes **(H)**, receptors for interferon ligands **(I)**, MHC class I molecules **(J)**, and proteasome subunit genes **(K)** in islet cells from allografts and syngeneic grafts.

and adhesion (**Figure 7C**). Mes-C3 was more enriched in allografts and characterized by high expression of *C1qa*, *Ctss*, *Laptm5*, and *Ly86*, indicating that these cells were active in antigen processing and presentation (**Figure 7C**). Thus, this subpopulation was possibly involved in immune responses. Mes-C4 represented another cluster that mainly originated from allografts. These cells expressed the pancreatic mesothelial cell marker genes *Upk3b*, *Krt19*, *Lrrn4*, and *Wt1*

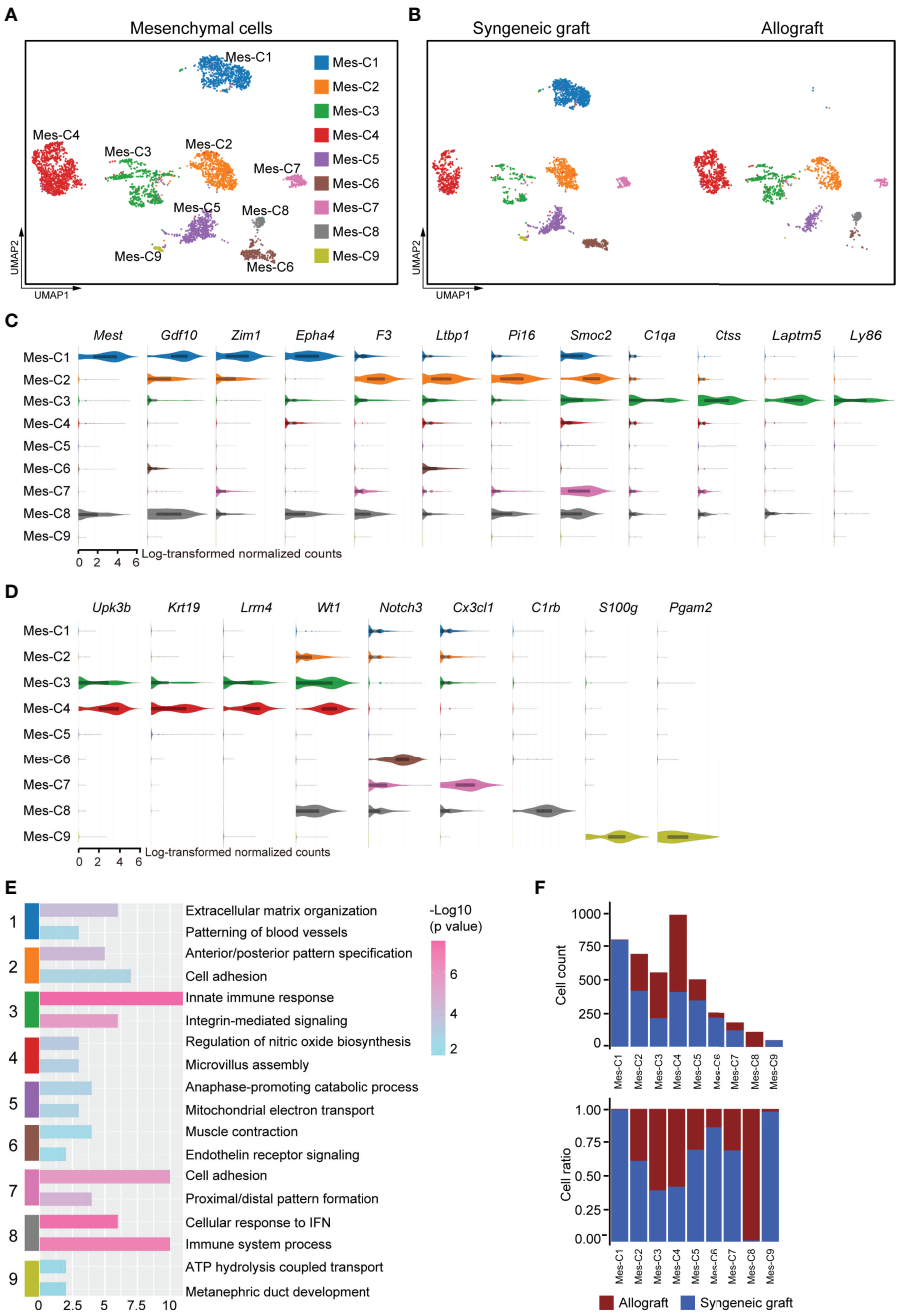

FIGURE 7 | Subclusters and molecular characteristics of mesenchymal cells 7 days post-transplantation. **(A, B)** UMAP visualization of mesenchymal cells, with each cell colorcoded for the associated cell subtype **(A)** and sample origin **(B)**. **(C, D)** Violin plots showing the smoothened expression distribution of the signature genes in subclusters C1-C3 **(C)** and C4-C9 **(D)**. **(E)** The top 2 gene ontology terms were based on marker genes in mesenchymal subclusters. **(F)** The number (upper panel) and a fraction (lower panel) of cells in the indicated mesenchymal cell subclusters.

(54, 55) **(Figure 7D)**. Mes-C6 expressed *Notch3*, a receptor regulator of cell differentiation and proliferation **(Figure 7D)**. Mes-C7 cells specifically expressed the chemokine *Cx3cl1*, and Mes-C8 cells highly expressed *C1rb*, which is involved in complement activation **(Figure 7D)**. Mes-C9 highly expressed *S100g* and *Pgam2* **(Figure 7D)**. Pathway analysis identified that cell adhesion and immune responses were functionally relevant to Mes-C2, Mes-C3, Mes-C7, and Mes-C8 **(Figure 7E; Supplementary Table 4)**. For all mesenchymal cells, Mes-C3, Mes-C4, and Mes-C8 were much more enriched in allografts, and the remaining clusters mainly contained cells from syngeneic grafts **(Figure 7F)**.

Cell-Cell Communication in Allograft

To further investigate the factors affecting the function of islet grafts, we systematically investigated ligand-receptor interactions across these cell types. Quantification of potential ligand-receptor interactions among all the cell types based on gene expression revealed strong interactions across these cell types, especially in mesenchymal cell-macrophage pairs, mesenchymal cell-islet cell pairs, Tconv-macrophage pairs, and CD8$^+$ T cell-

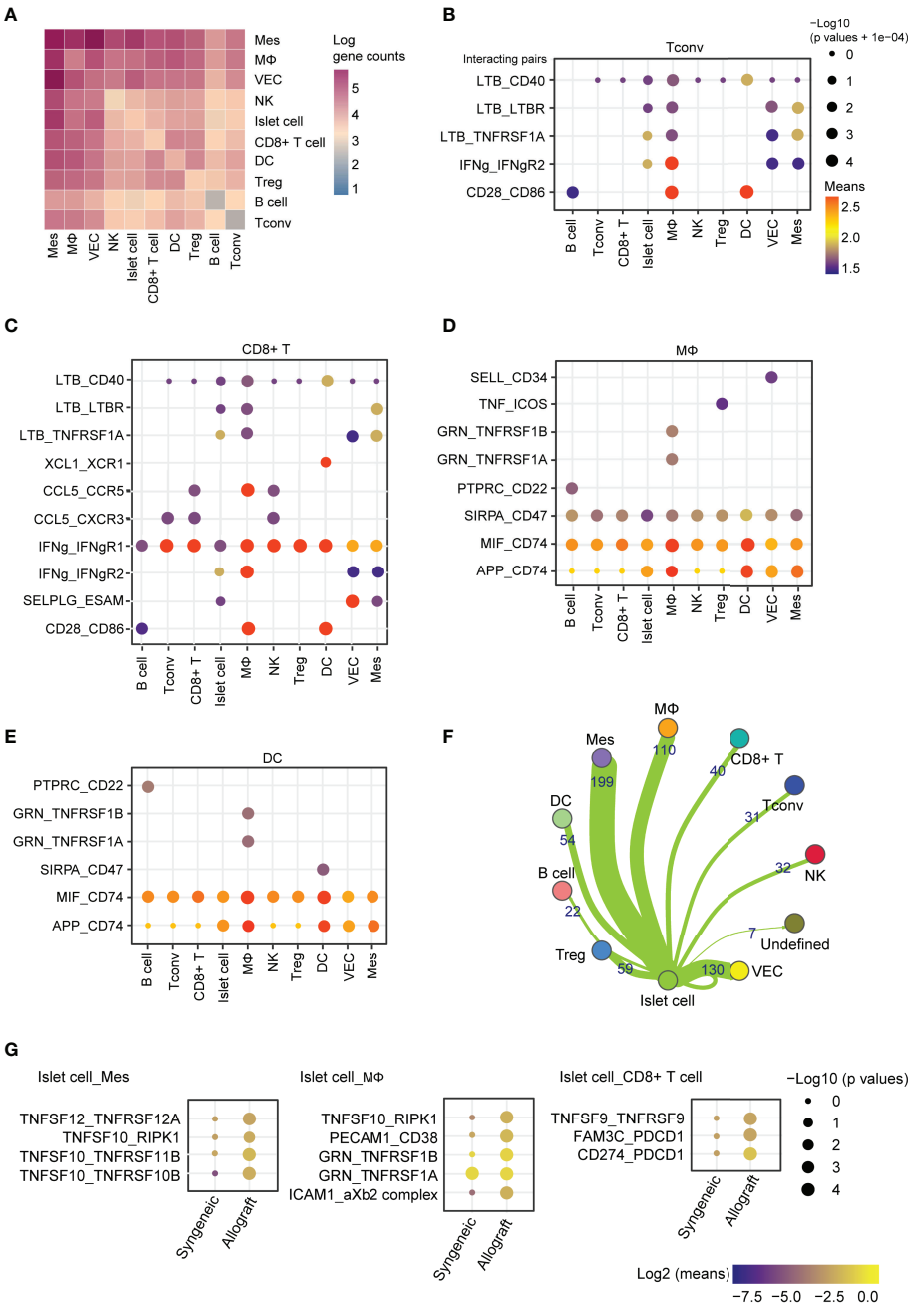

FIGURE 8 | Cell-cell communication between immune cells, mesenchymal cells, and islet cells in allografts. **(A)** Heatmap showing the log of the number of interacting gene pairs involved in cell-cell communications between every two cell types. **(B–E)** Dot plot showing ligand-receptor interactions of CD4$^+$ T cells **(B)**, CD8$^+$ T cells **(C)**, macrophages **(D)**, and dendritic cells **(E)** with other cell types. Color represents the means of the average gene expression level of interacting molecule one in cluster one and interacting molecule two in cluster two. Cell type labels were written as follows: cell type on the panel expressing the ligand and cell type below the panel expressing the receptor. **(F)** Network map showing cell-cell communications between islet cells and other cell types based on the number of genes involved. **(G)** Dot plot showing the ligand-receptor interaction of islet cell-expressing ligands and receptors expressed by mesenchymal cells, macrophages, and CD8$^+$ T cells. Color represents the maximum normalized log2 mean interaction score in each cell-cell pair, and size indicates the log of p values.

macrophage pairs (**Figure 8A**). To further reveal the variances of ligand-receptor interactions in allografts that were possibly related to graft immune rejection, we analyzed the differentially expressed genes of selected ligand-receptor pairs in allografts. *Lt*β and *Ifn*γ represented the most abundant ligands expressed by $CD4^+$ T cells and $CD8^+$ T cells for a vast range of receptors, including *Cd40*, *Lt*β*r*, *Tnfrsf1a*, *Ifngr1*, and *Ifngr2*, which were highly expressed by macrophages, vascular endothelial cells, islet cells, and mesenchymal cells (**Figures 8B, C**), indicating activated T cell responses in allografts. *Ccl5* and *Xcl1* expressed by $CD8^+$ T cells may also transduce signaling to macrophages and DCs through *Ccr5* and *Xcr*1, respectively (**Figure 8C**). Macrophages and DCs showed similar cell-cell communication patterns with other cell types through GRN_TNFRSF1, MIF_CD74, and APP_CD74 (**Figures 8D, E**), indicating an inflammatory state of these cells. Islet cells from allografts show strong interactions with mesenchymal cells and macrophages (**Figure 8F**). Elevated expression of *Tnfsf* members *Tnfsf12*, *Tnfsf10*, and *Tnfsf9* in islet cells mediated putative ligand-receptor interactions with mesenchymal cells, macrophages, and $CD8^+$ T cells (**Figure 8G**).

DISCUSSION

Islet transplantation to treat the late stage of T1DM patients has recently made inspiring success in clinical trials (5, 6). However, the overall characteristics of the immune microenvironment in islet allografts remain unclear. We used scRNA-seq to comprehensively analyze the microenvironment in islet grafts using a mouse model of allografts (BALB/c islets to C57BL/6 recipients) and syngeneic grafts (C57BL/6 islets to C57BL/6 recipients). To obtain intact immune infiltrates, we abandoned any immunosuppressive agents. In mouse model of allogeneic islet transplantation, acute rejection usually occurs within two weeks posttransplant (13.8 ± 2.7 days) (56, 57). We, therefore, collected the grafts 7 days post-transplantation, because it was hard to harvest the allograft samples at later time point (14-day) (**Supplementary Figure 1B**). Our data then recovered T lymphocytes and myeloid cells as the main components of grafts 7 days post-transplantation, especially in allografts. As a matter of concern in immune cell abundance, T cells (including CD4+ and CD8+ T cells) and macrophages accounted for 58% of the total cellularity. Other immune cells, including DCs, NKs, and B cells, were detected in grafts but with low abundance.

Our results provide several novel insights into mouse islet allograft rejection. We revealed the heterogeneity of $CD4^+$ T cells (Tconv and Treg) and activated cytotoxic $CD8^+$ T cells, dividing T cells, and activated NKT cells. All T cell subclusters were significantly enriched in allografts compared with syngeneic grafts, consistent with previous studies showing that T lymphocytes are the main mediator of transplant rejection. Among these, $CD8^+$ T cells exhibited an activated state that expressed *Ifn*γ, *Ifn*β, and *Xcl1* and receptors *Ccr7* and *Cxcr3*. We also identified increased regulatory T cells in allografts, which may reflect feedback control of excessive immune responses in allografts. A small population of cytotoxic $CD8^+$ T cells was identified in islet allografts, indicating the recruitment of host immature T cells by allogeneic islet cells. However, the function of the subpopulation needs to be further characterized.

Macrophages mediate the first phase of the immune response post-transplantation, representing the majority of cells in the transplanted organ during episodes of severe rejection (58). We detected an enrichment of M2 macrophages in syngeneic grafts with high expression of M2-associated genes such as *Igf1*, *Pf4*, *Gdf15*, *Ms4a7*, *Trem2*, *Mrc1*, and *Csf1r* in this population. These results indicated that macrophages may be required for the maintenance of exogenous islet grafts. In contrast, allograft infiltrating macrophages were inflammatory and activated, as they highly expressed the C-X-C motif chemokines *Cxcl9*, *Cxcl10*, and complement *C3*. These macrophages also expressed genes encoding molecules involved in antigen presentation and processing and chemotaxis, reflecting their roles in mediating immune responses.

As expected, we recovered rare islet cells from allografts due to immune rejection. Comparison of the gene expression features in islet cells from allografts with those from syngeneic grafts identified that the allogeneic islet cells were activated by interferons with the evidence of upregulated IFN-inducible genes and interferon ligand receptors. The high expression of *Ifn*γ by activated T cells may contribute well to the activation of allogeneic islet cells. In addition, allogeneic islet cells highly expressed MHC class I molecules and genes involved in MHC class I-mediated antigen presentation. Thus, these results indicated that allogeneic islet cells were transformed into antigen-presenting cell-like cells. This transformation may dramatically facilitate the interaction with cytotoxic $CD8^+$ T cells and promote the destruction of islet allografts.

There are several limitations of this study. Firstly, we only analyzed 7 days post-transplant. Depending on the model, an additional time point later in rejection (14 or 21-day time point) would allow for analysis of memory adaptive immune responses that would better mirror those that occur in transplant rejection. Secondly, there was a lack of analysis of circulating cells to contrast with those in islets. The addition of sequencing and/or FACS on lymph nodes, spleen, and PBMCs would be important to compare with immune populations and molecules of interest to those within grafts to ensure not due to mouse strain-specific differences. Thirdly, this study only included a small number of sequenced cells and from a limited number of mice. Fourthly, only male mice were used in this study.

In summary, we revealed the microenvironment in mouse islet syngeneic grafts and allografts, including three major cell populations (T cells, macrophages, and mesenchymal cells) and five minor cell populations (DCs, NKs, B cells, VECs, and islet cells). More importantly, we identified previously unknown microenvironment variations between islet syngeneic grafts and allografts: (1) The comprehensive landscape of $CD4^+$ T cells (Tconv and Treg), activated cytotoxic $CD8^+$ T cells, dividing T cells and NKT cells; (2) the decreased proportion of $Fcrl^+Igf1^+Stabl1^+$ and the increased proportion of $Cxcl9^+Cxcl10^+C3^+$ macrophage subpopulation, respectively; and (3) the combined M1 and M2 gene features in macrophages of islet grafts are different from conventional M1/M2 classification. Our mouse islet

microenvironment landscape provides a powerful resource for the identification of previously unknown cell subpopulations in allografts and syngeneic grafts, which suggests that these cells may contribute to the immune rejection of islet allografts.

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: Gene Expression Omnibus, accession number GSE198865.

ETHICS STATEMENT

The animal protocols were approved by the Institutional Biomedical Research Ethics Committee of Guangdong Medical University.

AUTHOR CONTRIBUTIONS

PC and LM initiated the study. FY and YL performed the analysis. YP performed mouse islet transplantation and sample collection. JD prepared the figures. SZ, ZW, JC, KD, QL revised the manuscript. PC and LM wrote the manuscript. ZP, and LM designed the study and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.853349/full#supplementary-material

Supplementary Figure 1 | Blood glucose levels and islets in transplanted mice. **(A)** Blood glucose levels of streptozotocin-induced C57BL/6 diabetic mice were followed for 7 days after syngeneic or allogeneic transplantation. Transplantations were performed on day 0. **(B)** Representative image of a graft section stained with antibodies specific for Insulin (red) and DAPI nuclear counterstain (blue). Syngeneic islets graft (C57BL/6 islets to C57BL/6 recipients) and allogeneic islets graft (BALB/c islets to C57BL/6 recipients) were harvested 14 days post-transplantation.

Supplementary Figure 2 | Overview of the cell components in mouse islets by single-cell RNA-seq. **(A, B)** UMAP visualization of the profile of the total cell, with each cell colorcoded for the associated cell type (upper panel) and the cell count of the indicated cell type (lower panel) (**A**, GSE84133_GSM2230761, ICR islets; **B**, GSE84133_GSM2230762, C57BL/6 islets). **(C, D)** UMAP visualization shows the expression of marker genes for delta cell (*Sst*), stellate (*Rgs5*), beta-cell (*Ins*), endothelial cell (*Pecam1*), alpha cell (*Gcg*), and macrophage (*Cd68*).

Supplementary Figure 3 | Gene expression signatures of T cell subclusters. **(A)** Statistical analysis of DEGs in $CD8^+$ T cell, $CD4^+$ Tconv, and Treg. **(B)** Gene ontology analysis of upregulated genes in each T cell subcluster. **(C)** Heatmap of row-scaled expression of marker genes for T cell activation within defined populations. Tconv, conventional T cell; Treg, regulatory T cell.

Supplementary Figure 4 | Subclusters and molecular characteristics of myeloid cells infiltrated in islet grafts. **(A)** UMAP visualization shows the expression of marker genes for myeloid cells. **(B)** Heatmap showing the expression of differentially expressed genes (DEGs) within 6 subclusters of myeloid cells. **(C)** Violin plots showing the smoothened expression distribution of *Pf4*, *Ms4a7*, *Mrc1*, *Gdf15*, *Trem2*, and *Csf1r* in each macrophage subcluster. **(D)** Heatmap showing the row-scaled expression of M2 macrophage feature gene expression within 3 subclusters of macrophages. **(E)** Violin plots showing the smoothened expression distribution of *Itgae*, *Itgax*, and *Itgam* in each dendritic cell subcluster.

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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

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Immune rejection is the major limitation for porcine xenograft survival in primate recipients. Proinflammatory cytokines play important roles in immune rejection and have been found to mediate the pathological effects in various clinical and experimental transplantation trials. IL-17 and TNF-α play critical pathological roles in immune disorders, such as psoriasis and rheumatoid arthritis. However, the pathological roles of human IL-17 (hIL-17) and human TNF-α (hTNF-α) in xenotransplantation remain unclear. Here we found that hIL-17 and hTNF-α additively or synergistically regulate the expression of 697 genes in porcine aortic endothelial cells (PAECs). Overall, 415 genes were found to be synergistically regulated, while 282 genes were found to be additively regulated. Among these, 315 genes were upregulated and 382 genes were downregulated in PAECs. Furthermore, we found that hIL-17 and hTNF-α additively or synergistically induced the expression of various proinflammatory cytokines and chemokines (*e.g.*, IL1α, IL6, and CXCL8) and decreased the expression of certain anti-inflammatory genes (*e.g.*, IL10). Moreover, hIL-17 plus hTNF-α increased the expression of IL1R1 and IL6ST, receptors for IL1 and IL6, respectively, and decreased anti-inflammatory gene receptor expression (IL10R). hIL-17 and hTNF-α synergistically or additively induced CXCL8 and CCL2 expression and consequently promoted primary human neutrophil and human leukemia monocytic cell migration, respectively. In addition, hIL-17 and hTNF-α induced pro-coagulation gene (SERPINB2 and F3) expression and decreased anti-coagulation gene (TFPI, THBS1, and THBD) expression. Additionally, hIL-17 and hTNF-α synergistically decreased occludin expression and consequently promoted human antibody-mediated complement-dependent cytotoxicity. Interestingly, hTNF-α increased swine leukocyte antigen (SLA) class I expression; however, hIL-17 decreased TNF-α-mediated

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SLA-I upregulation. We concluded that hIL-17 and hTNF-α likely promote the inflammatory response, coagulation cascade, and xenoantibody-mediated cell injury. Thus, blockade of hIL-17 and hTNF-α together might be beneficial for xenograft survival in recipients.

Keywords: xenotransplantation, immune rejection, cytokines, IL-17, TNF-α, PAECs, inflammation, coagulation

INTRODUCTION

Organ transplantation is an effective way of end-stage organ failure therapy. However, the shortage of human donors is a major limitation that prevents clinical application. Xenotransplantation is a promising way to solve this problem (1). Pigs are considered to be the most suitable organ donor animal (2, 3), and gene-modified pigs lead to increased xenograft survival; however, immune rejection is still a major hurdle in the survival of xenografts in primate recipients (4–10).

In pig-to-human organ transplantation, porcine vascular endothelial cells (ECs), which are the first cells to interact with the human immune system, play a critical role in the immune rejection of xenografts (11). Porcine ECs are activated by human-derived cytokines or chemokines and attacked by the human immune system in pig-to-human xenotransplantation (12). EC injury and dysfunction are critical for the inflammation and coagulation response, which will decrease pig organ survival in human recipients (13).

Cytokines play critical roles in inflammatory responses. In xenotransplantation, many proinflammatory cytokines are produced, including IL-17 and TNF-α (14). We previously found that human IL-6, IL-17, IL-1β, and TNF-α obviously activated porcine ECs, whereas human IFN-γ did not activate porcine ECs (12). Human TNF-α largely activates the NF-κB and mitogen-activated protein kinase (MAPK) pathways and induces downstream proinflammatory and procoagulation gene expression in porcine aortic endothelial cells (PAECs) (12). Human IL-17 also activates the NF-κB and MAPK pathways in PAECs (12). Several studies have reported that IL-17 and TNF-α additively or synergistically induce the expression of many genes to promote the development of various diseases, such as psoriasis and rheumatoid arthritis (15, 16). We also found that hIL-17 and hTNF-α additively or synergistically induce E-selectin, ICAM-1, IL-6, CXCL8, and CCL2 expression in PAECs (12). However, whether human IL-17 and TNF-α additively or synergistically induce the expression of certain genes to promote immune rejection in xenotransplantation remains unclear.

To answer this question, we stimulated PAECs with hIL-17, hTNF-α, or hIL-17 plus hTNF-α *in vitro*. We checked the mRNA levels in hIL-17- or hTNF-α-treated PAECs *via* transcriptome sequencing and analyzed the data using bioinformatics tools. We found that hIL-17 and hTNF-α additively or synergistically regulate the expression of hundreds of genes in PAECs. Many cytokines and chemokines (and some receptors for these genes) are regulated by IL-17 plus TNF-α. IL-17 plus TNF-α synergistically and additively induced CXCL8 and CCL2 expression and consequently promoted human neutrophil and THP-1 cell migration, respectively. Moreover, hIL-17 and hTNF-α additively or synergistically induced procoagulation gene (SERPINB2 and F3) expression and decreased anti-coagulation gene (TFPI, THBS1, and THBD) expression. Human IL-17 and hTNF-α also decreased occludin expression and consequently promoted human antibody-mediated complement-dependent cell injury. Here we demonstrate that hIL-17 and hTNF-α likely promote xenograft rejection in xenotransplantation.

MATERIALS AND METHODS

Reagents

Recombinant human IL-17, recombinant human TNFα, and recombinant porcine IFN-γ were purchased from R&D Systems (Minneapolis, MN, USA). Anti-actin antibody was purchased from Cell Signaling Technology (Boston, MA, USA), anti-occludin antibody was obtained from Thermo Fisher Scientific (Rockford, IL, USA), anti-FITC-labeled SLA class I antibody (SLA-I) was obtained from Bio-Rad (Hercules, CA, USA), and Cell Counting Kit-8 (CCK8) was purchased from Dojindo Laboratories (Kumamoto, Japan). The CCR2 (CCL2 receptor)-specific inhibitor RS504393 was from MedChemExpress (Shanghai, China).

Preparation of Porcine Aortic Endothelial Cells

PAECs were isolated from wild-type or *GGTA1/CMAH* double-knockout (DKO) Chinese domestic miniature Wuzhishan pig arteries as previously described (17). In brief, freshly harvested porcine arteries were treated with 0.05% collagenase B (Roche Applied Science, Indianapolis, IN, USA). We collected the cells and washed them with washing medium [RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS)]. The isolated PAECs were cultured with endothelial cell medium containing 10% (vol/vol) FBS (cat. no. 0025), 1% (vol/vol) penicillin/streptomycin solution (P/S, cat. no. 0503), and 1% (vol/vol) endothelial cell growth supplement (ECGS, cat. no. 1052) at 37°C with 5% CO_2. The culture medium was purchased from Sciencell (San Diego, CA, USA).

Western Blotting

PAECs were harvested after washing with ice-cold phosphate-buffered saline (PBS), lysed for 30 min in ice-cold RIPA lysis buffer supplemented with 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Roche, Indianapolis, IN, USA), and separated *via* 10% SDS–PAGE. After transfer onto polyvinylidene fluoride (PVDF)

membranes (Millipore, Billerica, MA, USA), the proteins on the PVDF membranes were blocked using 5% non-fat dried milk dissolved in TBST (20 mM Tris-HCl, 150 mM NaCl, pH 7.6) buffer supplemented with 0.1% (vol/vol) Tween 20 at room temperature for 1 h. After washing, the PVDF membranes were incubated with primary antibody overnight at 4°C and then washed with TBST. After incubation with secondary antibody for 1 h at room temperature, the blots were visualized with enhanced chemiluminescence detection reagents (Millipore).

Real-Time PCR

The procedure for real-time PCR has been reported previously (18). Briefly, total RNA was extracted from cells or tissues with TRIzol® Reagent (Invitrogen, Shanghai, China). cDNA samples were synthesized with PrimeScript™ RT Master Mix (Takara Bio, Dalian, LN, China). The levels of the genes of interest were quantified using TB Green® *Premix Ex Taq*™ (Tli RNaseH Plus) (Takara Bio). The expression level of the genes was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the rpl13a expression level. cDNA amplification was performed using a ViiA 7 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), and the oligonucleotide primer sequences are shown in **Supplementary Table S1**.

Flow Cytometry Analysis

SLA-I binding was assessed using flow cytometry as previously reported (12). Porcine aortic endothelial cells were collected and washed once with PBS, and 1×10^6 cells were resuspended in 100 µl PBS containing 1% BSA and then stained with FITC-labeled SLA-I antibody. Isotype-matched antibody was used as a negative control. After incubation for 30 min at 4°C in the dark, the cells were washed once with PBS, resuspended in 100 µl PBS containing 1% BSA and detected with a BD FACS Aria II flow cytometer (Franklin Lakes, NJ, USA). The extent of SLA-I binding to PAECs was evaluated by geometric mean fluorescence intensity (Gmean).

Human Antibody-Mediated Complement-Dependent Cytotoxicity

The protocol for human antibody-mediated complement-dependent cytotoxicity assessment has been previously reported (11). In brief, PAECs (4×10^3) were seeded into 96-well plates and treated with medium (as a negative control), hIL-17 (100 ng/ml), hTNF-α (2 ng/ml), or hIL-17 plus hTNF-α for 48 h. The supernatant was removed and replaced with RPMI-1640 medium containing 20% pooled human serum [experimental group—the serum was pooled from several healthy volunteers (n = 20), including all ABO blood types] or 20% heat-inactivated human serum (control group) for 2 h. After 2 h, CCK8 assays were used to assess the viability of PAECs. The supernatant was removed and replaced with RPMI-1640 medium containing 10% CCK8 reagent for 2 h. At 2 h later, the absorbance values of the wells at OD450 were measured using a multiscan GO spectrophotometer (Thermo Fisher, Vantaa, Finland). The percentage of cell death (cytotoxicity) was calculated according to the following formula:

$$\%\ \text{cytotoxicity} = (\text{OD of control group} - \text{OD of experimental group}) / \text{OD of control group} \times 100$$

Transwell Assay

The chemotaxis assay procedure has been reported previously (19). In brief, isolation of human neutrophils using a human neutrophil isolation kit was performed according to the manufacturer's instructions. The chemotaxis assay was performed in 24-well plates using 6.5 µm (for THP-1) or 3 µm (for human neutrophils) transwell inserts with 5-mm pore polycarbonate membranes (Corning). The PAECs were treated with hIL-17 (100 ng/ml), hTNF-α (2 ng/ml), or hIL-17 plus hTNF-α for 48 h, the supernatant was collected and then added to the lower transwell chamber, and 25×10^4 THP-1 or human neutrophils were seeded in the upper chamber. The transmigration assay was performed for 2 h at 37°C. For CCR2 inhibitor assay, THP-1 cells were treated with 10 µM RS504393 for 1 h and then seeded in the upper chamber. The membranes containing the migrated cells were carefully excised. Images were obtained with a microscope, and the migrated cells were counted. The migration data are presented as the number of migrating cells/field.

Transcriptome Sequencing

PAECs were treated with rhIL-17 (100 ng/ml), rhTNF-α (2 ng/ml), or rhIL-17 plus rhTNF-α for 0 or 6 h. RNA was extracted from the treated PAECs using TRIzol reagent (Invitrogen) and was utilized to construct the final library. The sequencing library was generated using the VAHTS mRNA-seq v2 Library Prep Kit for Illumina® (Vazyme, NR601, Nanjing, JS, China) following the manufacturer's recommendation. Library concentration was measured using a Qubit® RNA Assay Kit and Qubit® 3.0 for preliminary quantification. Insert size was assessed using an Agilent Bioanalyzer 2100 system, and after the insert size was consistent with expectations, the qualified insert size was accurately quantified *via* qPCR using a Step One Plus Real-Time PCR system (Applied Biosystems). Clustering of the index-coded samples was performed on a cBot Cluster Generation System (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. After cluster generation, the library preparations of a total of 12 samples were sequenced on an Illumina HiSeq X Ten platform using a 150-bp paired-end module.

Whole-transcriptome sequencing data were filtered and mapped to the porcine genome. The differentially expressed gene-seq method was based on a Poisson distribution (fold change >1.5 and adjusted P-value <0.05). Additive genes and synergistic genes (ASGs) were screened according to the definition. The whole-transcriptome sequencing data can be found in NCBI (https://dataview.ncbi.nlm.nih.gov/object/PRJNA779585?reviewer=3areeogpjo6vkvlr0i3hhb1s64) under accession number PRJNA779585.

According to the Kyoto Encyclopedia for Genes and Genomes (KEGG) annotation results and the official classification, we separately classified the functional and biological pathways of the differentially expressed genes and used R software for enrichment analysis. Bubble charts, heat maps, and volcano maps were generated to visualize the differentially expressed genes on the basis of log-normalized expression values of significant genes using v3 R software.

Definition of Additive or Synergistic Genes

The additive genes and synergistic genes were defined according to a previous report (15). A gene was considered to be synergistically induced by IL-17/TNF-α when the combined effect was greater than the sum of the separate effects. A gene was considered to be additive when the combined effect was greater than the two individual effects but lower than the sum of the separate effects. In brief, we defined (x) to represent the $\log_2$-FCH (fold change) induced in hIL-17-treated PAECs (y) to represent the $\log_2$-FCH induced by hTNF-α and ($x + y$) to represent the $\log_2$-FCH induced by the combination of hIL-17 and hTNF-α in treated PAECs. Two tests were then used to define whether a gene was synergistic or additive, and the gene was excluded if it was antagonistic and induced by hIL-17 and hTNF-α. The flow chart was shown in **Supplementary Figure S1**. First, we used hypothesis testing to test whether the effect regulated by the two cytokines together was different than the sum of the individual effects, *i.e.*:

$$\text{Test 1}: \text{Ho}:(x+y) = (x) + (y) \quad \text{vs.} \quad \text{Ha}:(x+y) \neq (x) + (y)$$

Then, we tested whether the combined effect was different than both individual effects, *i.e,*:

$$\text{Test 2}: \text{Ho}:(x+y) = (x) \quad \text{vs.} \quad \text{Ha}:(x+y) \neq (x) \text{ and Ho}:(x+y) = (y) \quad \text{vs.} \quad \text{Ha}:(x+y) \neq (y)$$

Synergistic Genes

A gene was considered synergistic if it passed test 1 and $|(x + y)| > |(x) + (y)|$. A synergistic increase is the difference $|(x + y)| - |(x) + (y)|$. Synergism can be either positive (if the synergist increase is positive) or negative.

Additive Genes

A gene was considered additive (if it is not synergistic) if the combined effect was greater than both individual effects, *i.e.*, the gene passes test 2 for both x and y.

The synergistic genes are highlighted in red, and the additive genes are shown in black in **Supplementary Tables S2, S3** (Ho: the null hypothesis; Ha: the alternative hypothesis).

Statistical Analysis

Experimental data are presented as mean ± SEM. GraphPad Prism 5 software is used to perform statistical analysis and graph development. Statistical significance between the groups was calculated using two-tailed Student's *t*-test using Microsoft Office Excel software. p-values <0.05 were considered significant.

RESULTS

Human IL-17 and TNF-α Synergistically Regulated the Expression of Various Immune-Related Genes in PAECs

Human IL-17 plus hTNF-α additively or synergistically regulated the expression of 697 genes in PAECs (**Figure 1A**, **Supplementary Tables S2, S3**). A total of 315 genes were upregulated in PAECs, 166 of which were synergistically induced, while another 149 genes were additively induced (**Supplementary Table S2**). A total of 382 genes were downregulated in PAECs, 249 of which were synergistically regulated, while 133 other genes were additively regulated (**Supplementary Table S3**). These ASGs were subjected to KEGG pathway enrichment analysis and annotation. Many immune-related signaling pathways, such as the TNF-α, IL-17, MAPK, and Toll-like receptor signaling pathways, were obviously enriched (**Figure 1B**). In addition to these pathways, the cytokine–cytokine receptor interaction was also enriched (**Figure 1B**). The KEGG pathway annotation analysis demonstrated that 159 genes were related to signal transduction (**Supplementary Figure S2**). Among the organismal systems, the immune system represented the top enrichment, involving 86 genes (**Supplementary Figure S2**), suggesting that hIL-17 plus hTNF-α primarily activated immune-related genes in PAECs. Cytokines and chemokines play critical roles in xenograft rejection, and we found that various proinflammatory cytokines, such as IL1α and IL6, and chemokines, including CCL2, CCL11, CXCL8, and CXCL2, were upregulated (**Figure 1C**). In contrast, the anti-inflammatory gene IL10 was downregulated (**Figure 1C**). The ligand–receptor analysis identified that CCL11, IL1α, IL6, IL11, and their receptors were upregulated, while IL-10-IL10RB and KITLG-KIT were obviously downregulated (**Figure 1D**). We used RT-PCR to validate the expression of several cytokines and chemokines and found that CCL20, CSF3, IL11, and CXCL2 were slightly induced by IL-17 or TNF-α alone but dramatically induced by IL-17 plus TNF-α, suggesting that these genes were synergistically induced (**Figure 2**). CCL11 and IL1α were additively induced by IL-17 plus TNF-α (**Figure 2**). These results were consistent with the transcriptome sequencing data (**Supplementary Table S2**). Collectively, the data suggest that hIL-17 and hTNF-α synergistically induce proinflammatory cytokine and chemokine expression to amplify the inflammatory response.

Due to several gene-modified pigs used for xenotransplantation, we asked whether human IL-17 and TNF-α could synergistically or additively induce proinflammatory cytokine and chemokine expression in PAECs from gene-modified pigs. We isolated PAECs from α1,3-galactosyltransferase (GGTA1) and cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) gene double-deficient pigs. The two genes are responsible for the synthesis of Galα-1,3-Gal (Gal) and N-glycolylneuraminic acid (Neu5Gc), two carbohydrate xenoantigens that are respectively important for xenotransplantation, and the *GGTA1/CMAH* DKO pigs could largely reduce immune rejection in xenotransplantation.

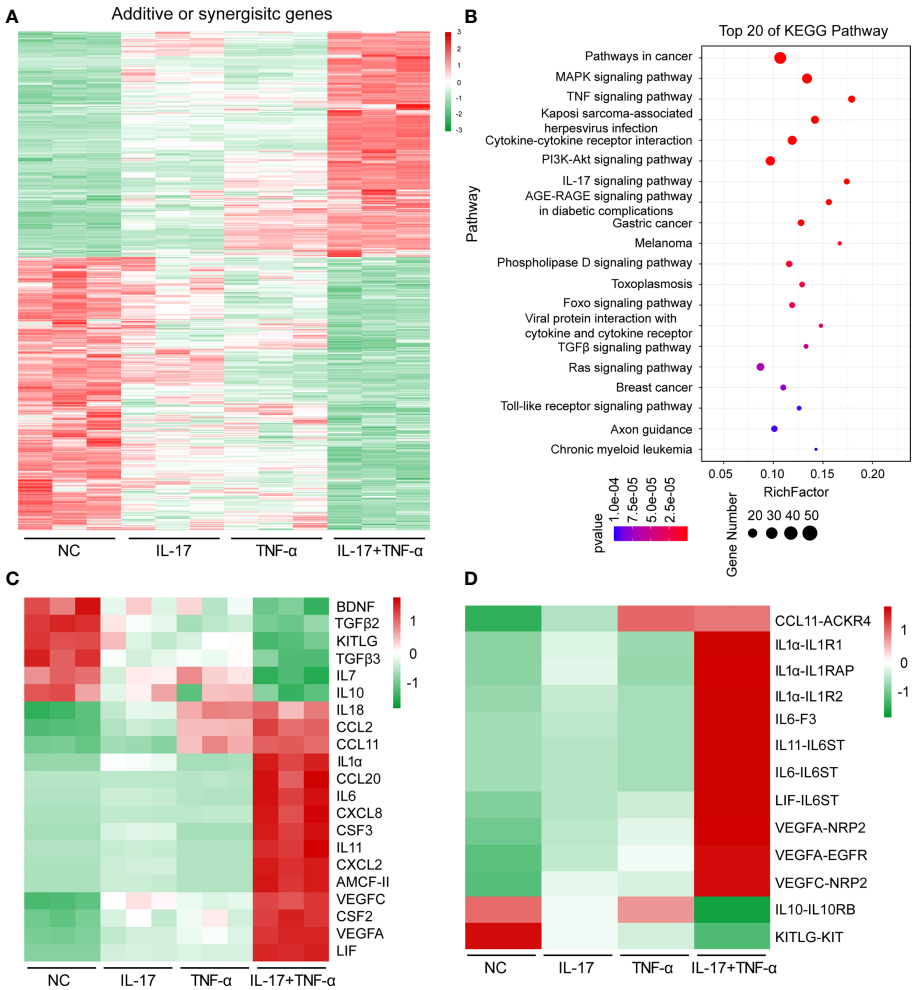

FIGURE 1 | Human IL-17 and TNF-α additively or synergistically induced the expression of hundreds of genes in porcine aortic endothelial cells. **(A)** Heat map of additive or synergistic genes (ASGs) in the control group, IL-17 group, TNF-α group, and IL-17 plus TNF-α group. **(B)** Top 20 enriched Kyoto Encyclopedia for Genes and Genome pathways of ASGs between the control group and the IL-17 plus TNF-α group. **(C)** Heat map of regulated cytokine or chemokine gene expression levels in the control group, IL-17 group, TNF-α group, and IL-17 plus TNF-α group. **(D)** Ligand and receptor analysis of ASGs in the control group, IL-17 group, TNF-α group, and IL-17 plus TNF-α group.

We treated the *GGTA1/CMAH DKO* PAECs with hIL-17, hTNF-α, or hIL-17 plus hTNF-α and found that IL-17 plus TNF-α synergistically induced the expression of CCL20, CSF3, and CXCL2 and additively induced the expression of CCL11, IL1α, and CXCL8 (**Supplementary Figure S3**). The data suggest that human IL-17 and TNF-α synergistically or additively induce proinflammatory cytokine or chemokine expression not only in wild-type PAECs but also in *GGTA1/CMAH DKO* PAECs. Thus, IL-17 and TNF-α likely promote the rejection of *GGTA1/CMAH DKO* xenograft in xenotransplantation.

We also found that IL-17 and TNF-α additively or synergistically regulate the expression of 65 cell surface proteins (**Supplementary Figure S4**): 29 genes were upregulated, and 36 genes were downregulated. The regulated genes included several receptors, adhesion molecules, and tight junction genes, such as IL1R1, ICAM1, and occludin (OCLN). These genes might increase proinflammatory signaling, promote immune cell migration, and enhance xenoantibody-mediated complement-dependent cytotoxicity (CDC). Moreover, some of these regulated cell surface genes might be xenoantigens, which will be investigated in the future.

IL-17 Plus TNF-α Increased Neutrophil and Monocyte Chemotaxis

The transcriptome sequencing data and our previous study showed that hIL-17 and hTNF-α synergistically induce CXCL8 expression and additively induce CCL2 expression in PAECs (**Figure 1C** and **Supplementary Table S2**) (12). We also validated their expression levels *via* RT-PCR analysis (**Figure 3A**). The chemokines CXCL8 and CCL2 have neutrophil and monocyte chemotactic activity, respectively; thus, we asked whether the supernatant of hIL-17- or hTNF-α-

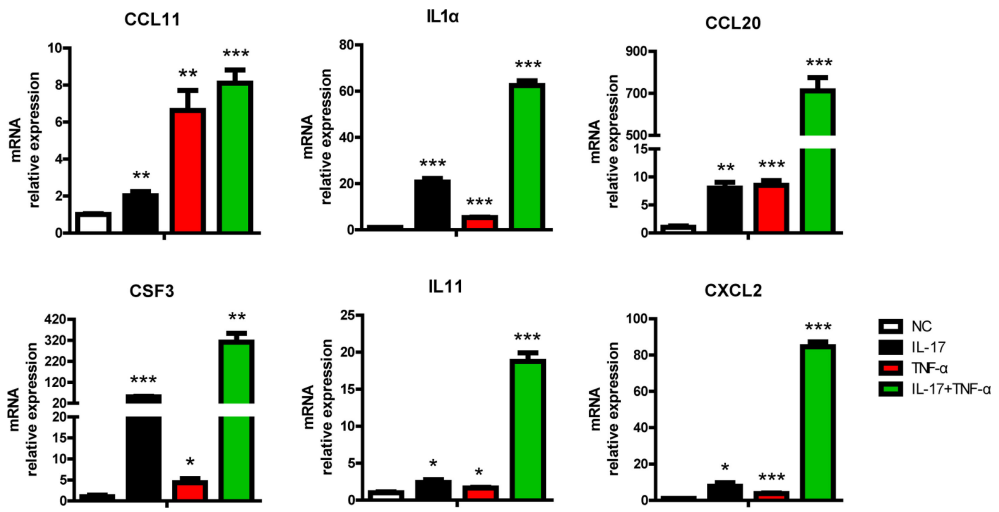

FIGURE 2 | IL-17 and TNF-α additively or synergistically induced chemokine or cytokine expression in porcine aortic endothelial cells (PAECs). PAECs were treated with rhIL-17 (100 ng/ml), rhTNF-α (2 ng/ml), or rhIL-17 plus rhTNF-α for 0 or 6 h. The induction of CCL11, IL1α, CCL20, CSF3, IL11, and CXCL2 mRNA was measured *via* real-time PCR. Data are representative of at least three independent experiments (mean ± SEM). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, determined by Student's *t*-test.

treated PAECs had monocyte and neutrophil chemotactic activity. We found that the supernatant of hIL-17- or hTNF-α-treated PAECs alone increased human neutrophil migration and that the supernatant of hIL-17 plus hTNF-α-treated PAECs had greater chemotactic activity than the supernatant of IL-17- or TNF-α-treated PAECs alone (**Figures 3B, C**). In a THP-1 (a human leukemia monocytic cell line) cell migration assay, the results were similar to those of human neutrophil migration (**Figures 3B, C**). In order to investigate whether IL-17 or TNF-α directly mediated THP-1 cell migration, we collected the supernatant from PAECs without IL-17 or TNF-α treatment and then added IL-17 plus TNF-α to the supernatant for THP-1 cell migration assay. We found that the supernatant with IL-17 plus TNF-α did not enhance THP-1 cell migration, suggesting that IL-17 or TNF-α cannot directly mediate THP-1 cell migration (**Supplementary Figure S5**). To confirm whether IL-17 plus TNF-α increased THP-1 cell migration through inducing CCL2 expression, we used RS504393, a specific inhibitor of CCR2 which is the receptor for CCL2, treated THP-1 cells, and found that RS504393 almost completely blocked THP-1 cell migration, which suggest that IL-17 plus TNF-α increases THP-1 cell chemotaxis through enhancing CCL2 expression (**Supplementary Figure S5**). Collectively, these data suggest that hIL-17 plus hTNF-α increases human neutrophil or monocyte chemotaxis and that the increased chemotaxis is likely due to increased CXCL8 or CCL2 production by PAECs.

IL-17 Plus TNF-α Regulated Coagulation-Related Gene Expression in PAECs

Previously, we found that hIL-17 plus hTNF-α increased the mRNA level of F3, a procoagulation gene, in PAECs (12). In the present study, we found that F3 was induced in PAECs treated with hIL-17 plus hTNF-α (**Figure 4A**). In addition to F3, we found that the mRNA level of another pro-coagulation gene (SERPINB2) was upregulated, while the mRNA levels of three anti-coagulation genes (TFPI, THBS1, and THBD) were downregulated in PAECs treated with hIL-17 plus hTNF-α (**Figures 4A, B**). To validate the data, we measured the mRNA levels of these genes in PAECs treated with hIL-17 or hTNF-α and found that SERPINB2 was increased, while TFPI, THBS1, and THBD were downregulated in PAECs treated with hIL-17 plus hTNF-α (**Figure 4C**). The mRNA levels of THBS1 and THBD were slightly downregulated by IL-17 or TNF-α alone and largely reduced by IL-17 plus TNF-α. Collectively, these data suggest that hIL-17 and hTNF-α amplify the coagulation reaction in response to xenografts through additive or synergistic effects.

IL-17 Plus TNF-α Promoted Human Antibody-Mediated Complement-Dependent Cytotoxicity in PAECs

Tight junction genes play an important role in xenoantibody-mediated CDC (20, 21). In the present study, we found that OCLN, ESAM, CLDN6, and CDH5 were downregulated and that JAM2, CLMP, BVES, and CLDN1 were upregulated in PAECs treated with hIL-17 plus hTNF-α (**Figure 5A**). Moreover, we found that hTNF-α alone decreased OCLN expression in PAECs and that the mRNA and protein levels of occludin were much lower in PAECs treated with hIL-17 plus hTNF-α than in PAECs treated with TNF-α alone (**Figures 5B, C**). We previously found that hTNF-α promoted human antibody-mediated CDC in porcine ECs by downregulating occludin expression; thus, we asked whether hIL-17 plus hTNF-α had stronger PAEC cytotoxicity in a human-mediated CDC model. We found that TNF-α alone exerted increased

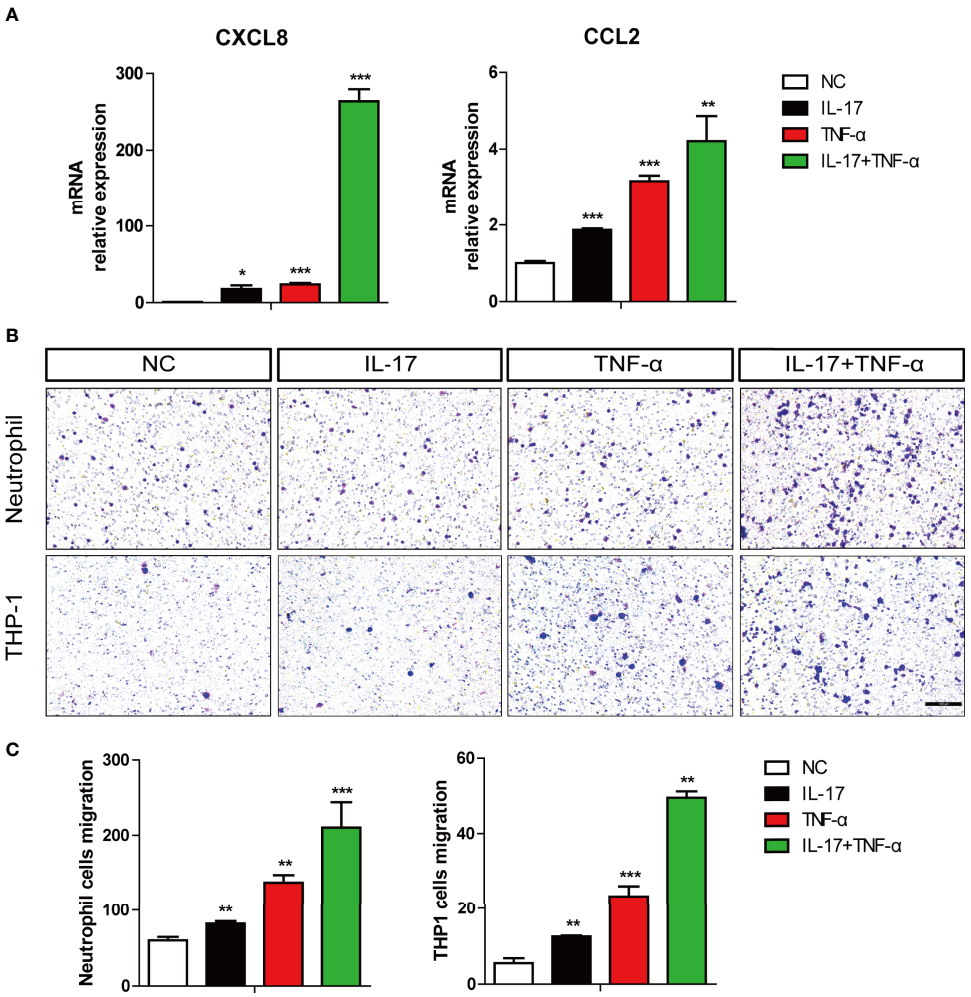

FIGURE 3 | The combination of IL-17 with TNF-α largely increased the chemotaxis of THP-1 cells and human neutrophils. **(A)** Porcine aortic endothelial cells (PAECs) were treated with rhIL-17 (100 ng/ml), rhTNF-α (2 ng/ml), or rhIL-17 plus rhTNF-α for 0 or 6 h. The induction of CXCL8 or CCL2 mRNA was measured *via* real-time PCR. **(B)** The PAECs were treated with IL-17, TNF-α, or IL-17 plus TNF-α for 48 h, and the supernatant was collected for chemotaxis assays. Human neutrophils or THP-1 cells were used to assess cell migration. **(C)** The number of migrating cells per field was determined as in **(B)**. Data are representative of at least three independent experiments (mean ± SEM). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, determined by Student's *t*-test.

PAEC cytotoxicity, while hIL-17 plus hTNF-α led to more PAEC death than TNF-α alone (**Figure 5D**). To exclude the direct cytotoxicity of hIL-17 or hTNF-α to PAECs, we treated PAECs with or without hIL-17, hTNF-α, or hIL-17 plus hTNF-α for 48 h and assess the viability of PAECs with CCK8. We found that the OD450 of the negative control group was almost equal to hIL-17, hTNF-α, or hIL-17 plus the hTNF-α treated group, suggesting that hIL-17 or hTNF-α does not directly affect the viability of PAECs (**Supplementary Figure S6**). These data suggest that hIL-17 and hTNF-α likely increase xenoantibody-mediated CDC, which leads to xenograft injury.

IL-17 Decreased TNF-α-Mediated SLA-I Upregulation in PAECs

We previously found that hTNF-α could induce SLA-I expression; therefore, we asked whether hIL-17 plus hTNF-α could additively or synergistically increase SLA-I expression (12). Surprisingly, we found that hTNF-α significantly induced SLA-I expression; however, hIL-17 almost completely blocked hTNF-α-mediated SLA-I upregulation (**Figure 6A**). We also found that hIL-1β increased SLA-I expression and that hIL-17 slightly reduced hIL-1β-mediated SLA-I upregulation (**Figure 6B**). Moreover, porcine IFN-γ (pIFN-γ) increased SLA-I expression, but IL-17 did not decrease IFN-γ-mediated SLA-I expression (**Figure 6C**). These data suggest that IL-17 has a suppressive effect on TNF-α- or IL-1β-mediated SLA-I upregulation.

IL-17 and IL-1β or IFN-γ Had Additive or Synergistic Effects in PAECs

Human IL-17 and hTNF-α additively or synergistically regulated the expression of various genes in PAECs, and we wondered whether hIL-17, in combination with hIL-1β or pIFN-γ, had

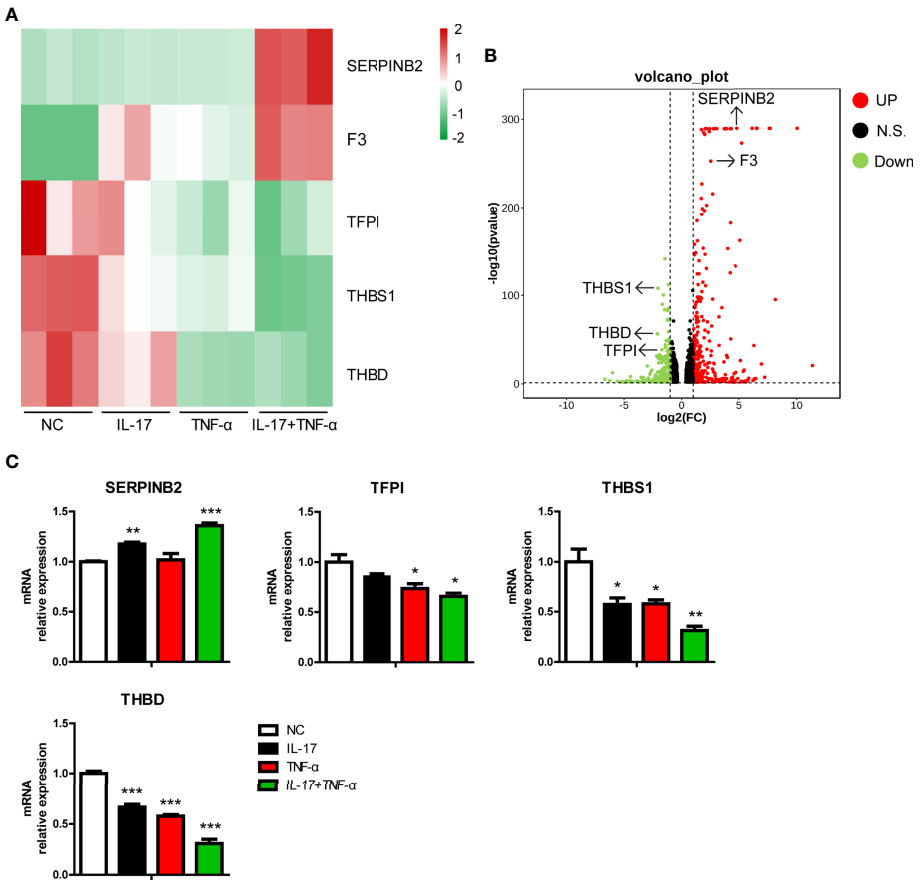

FIGURE 4 | IL-17 and TNF-α regulated coagulation-related gene expression in porcine aortic endothelial cells (PAECs). **(A)** Heat map showing regulated coagulation-related additive or synergistic genes (ASGs) in the control group, IL-17 group, TNF-α group, and IL-17 plus TNF-α group. **(B)** Volcano plots displaying regulated coagulation-related ASGs between the control group and the IL-17 plus TNF-α group. **(C)** The PAECs were treated with rhIL-17 (100 ng/ml), rhTNF-α (2 ng/ml), or rhIL-17 plus rhTNF-α for 0 or 6 h. The induction of SERPINB2, TFPI, THBS1, and THBD mRNA was measured *via* real-time PCR. Data are representative of at least three independent experiments (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, determined by Student's *t*-test.

additive or synergistic effects in PAECs. We found that hIL-17 and hIL-1β synergistically induced IL6 expression and additively induced E-selectin, ICAM-1, VCAM-1, CXCL8, CCL2, and CXCL2 expression (**Figure 7A**). Human IL-17 and pIFN-γ also synergistically induced IL6 expression and additively induced E-selectin, ICAM-1, VCAM-1, and CCL2 expression (**Figure 7B**). However, human IL-17 and pIFN-γ did not additively or synergistically induce CXCL8 or CXCL2 expression (**Figure 7B**). These data suggest that hIL-17 and hIL-1β or pIFN-γ can additively or synergistically induce the expression of specific proinflammatory cytokines, chemokines, and adhesion genes in PAECs.

DISCUSSION

We previously found that hIL-17 and hTNF-α can activate PAECs and induce downstream gene expression in PAECs (12). However, the pathological role of hIL-17 and hTNF-α in xenotransplantation is less well investigated. Here we found that hIL-17 and hTNF-α additively and synergistically regulate the expression of 697 genes in PAECs: 415 genes were synergistically regulated, and 282 genes were additively regulated. We found that hIL-17 plus hTNF-α increased the expression of many proinflammatory cytokines and chemokines and reduced the expression of specific anti-inflammatory genes. Moreover, we found that hIL-17 plus hTNF-α promoted human neutrophil and THP-1 migration by inducing CXCL8 and CCL2 expression. Human IL-17 plus hTNF-α increased procoagulation gene expression and decreased anti-coagulation gene expression. Human IL-17 plus hTNF-α increased human antibody-mediated CDC in PAECs. Based on our observations, we speculate that hIL-17 and hTNF-α have important pathological roles in promoting inflammation, the coagulation response, and xenoantibody-mediated cell injury in pig-to-primate xenotransplantation.

Several studies have reported that IL-17 and TNF-α have additive and synergistic effects in mouse and human systems (15, 16, 22, 23). The synergistic effect has been reported to promote the development of immune-related diseases, such as

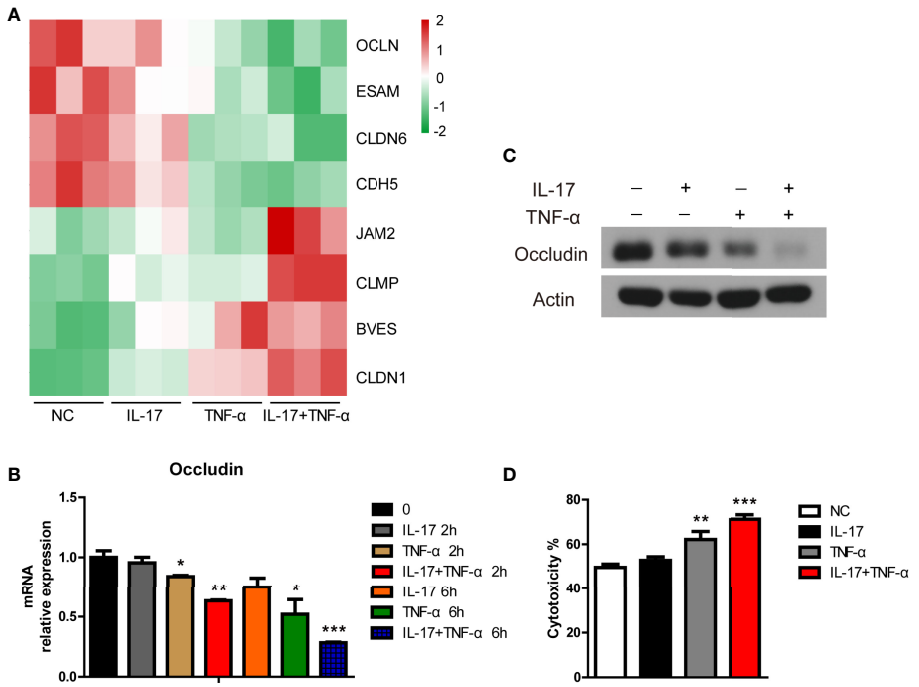

FIGURE 5 | IL-17 increased TNF-α-mediated cytotoxicity toward porcine aortic endothelial cells (PAECs) in a human antibody-mediated complement-dependent cytotoxicity model. **(A)** Heat map showing tight junction genes among the additive or synergistic genes in the control group, IL-17 group, TNF-α group, and IL-17 plus TNF-α group. **(B)** The PAECs were treated with rhIL-17 (100 ng/ml), rhTNF-α (2 ng/ml), or rhIL-17 plus rhTNF-α for 0, 2, or 6 h. The mRNA level of occludin was measured *via* real-time PCR. **(C)** The PAECs were treated with rhIL-17 (100 ng/ml), rhTNF-α (2 ng/ml), or rhIL-17 plus rhTNF-α for 24 h. The lysates were analyzed by western blotting with antibodies against occludin and actin. **(D)** The PAECs were treated with rhIL-17 (100 ng/ml), rhTNF-α (2 ng/ml), or rhIL-17 plus rhTNF-α for 24 h and then exposed to human serum to induce antibody-mediated CDC. Data are representative of at least three independent experiments (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, determined by Student's *t*-test.

psoriasis and rheumatoid arthritis (15, 16). Here we found that hIL-17 and hTNF-α synergistically regulate the expression of 415 genes in PAECs. The KEGG pathway annotation suggested that most of these genes are associated with the immune system. Human IL-17 and hTNF-α synergistically increased proinflammatory gene (such as CCL20, IL11, IL6, CXCL2, and CXCL8) expression and reduced anti-inflammatory gene (such as IL10) expression. Moreover, some receptors for these genes were also additively or synergistically regulated. The expression of IL6ST (receptor for IL11 or IL6) or F3 (receptor for IL6) was induced, while the expression of IL10RB (receptor for IL10) was decreased in PAECs treated with hIL-17 plus hTNF-α. These data suggest that hIL-17 and hTNF-α might amplify the immune response by synergistically increasing proinflammatory gene expression and decreasing anti-inflammatory gene expression in pig-to-human xenotransplantation.

The chemokines CXCL8 and CCL2 can recruit neutrophils and monocytes to the inflammatory site, respectively (24–27). The present study found that hIL-17 and hTNF-α synergistically increased CXCL8 expression and additively induced CCL2 expression in PAECs. The supernatant from hIL-17 plus hTNF-α-treated PAECs had much more chemotactic activity (to recruit human neutrophils and THP-1 cells) than the supernatant from hIL-17- or hTNF-α-treated PAECs. Thus, our study suggests that hIL-17 plus hTNF-α has the potential ability to recruit neutrophils and monocytes to inflammatory response sites to amplify the immune reaction.

The coagulation cascade is a tightly regulated process (28). In pig-to-primate xenotransplantation, the coagulation cascade is dysregulated, and this dysregulation of the coagulation cascade is a major obstacle for xenograft survival (29). F3 promotes the conversion of prothrombin to thrombin to initiate the extrinsic coagulation cascade (30). Previously, we found that hIL-17 plus hTNF-α increases F3 expression (12). In the present study, we found that hIL-17 plus hTNF-α significantly increased the expression of two procoagulation factors (SERPINB2 and F3) and decreased the expression of three anticoagulation factors (TFPI, THBS1, and THBD). Based on these data, we speculate that hIL-17 and hTNF-α likely promote the coagulation cascade and consequently decrease xenograft survival in xenotransplantation. To confirm the role of hIL-17 and hTNF-α in the coagulation response in xenotransplantation, we intend to design experiments to answer this question in the near future.

Xenoantibody-mediated complement-dependent cell killing is another limiting factor in xenograft survival. Human antibody-mediated CDC is a suitable *in vitro* model to mimic the process. The barrier function of porcine ECs is important for the protection of ECs in human antibody-mediated CDC. Tight junction genes are critical for barrier integrity (31). Dalmasso et al. reported that

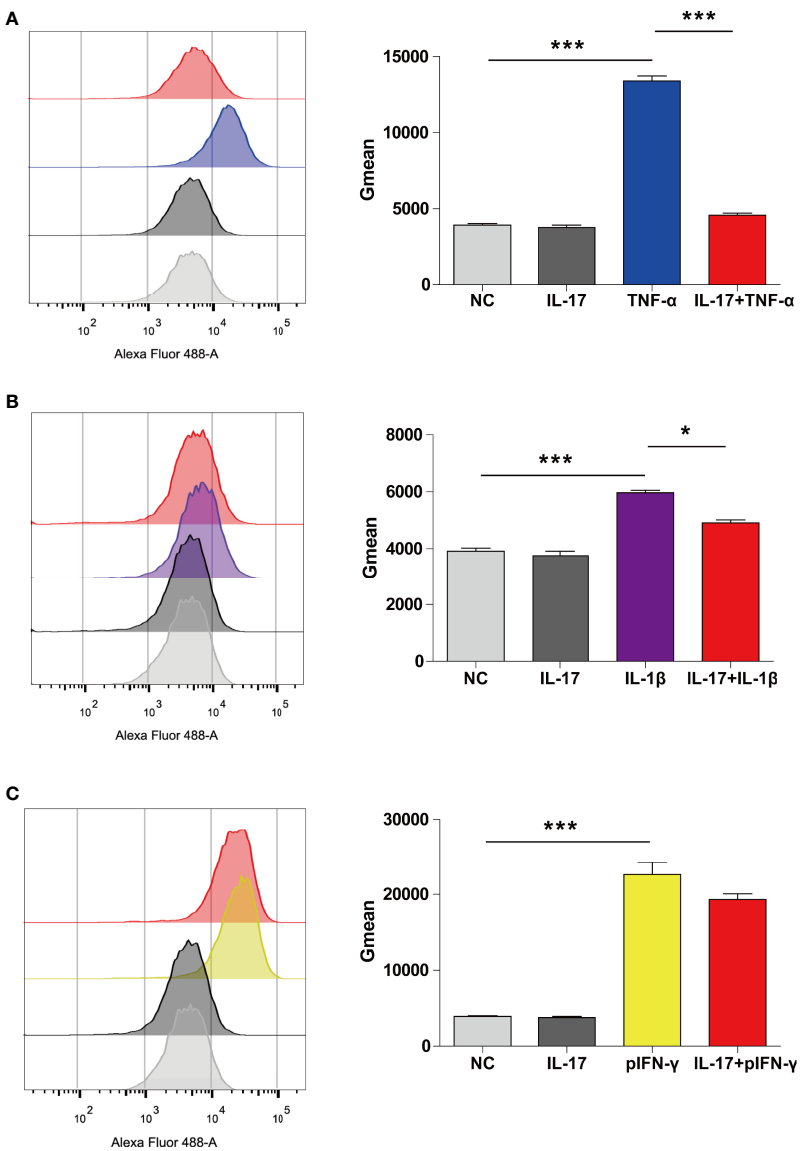

FIGURE 6 | IL-17 decreased TNF-α- or IL-1β-induced SLA-I expression in porcine aortic endothelial cells (PAECs). **(A)** The PAECs were untreated or treated with rhIL-17 (100 ng/ml), rhTNF-α (2 ng/ml) or rhIL-17 plus rhTNF-α for 24 h, and the expression of SLA-I was measured *via* flow cytometry. **(B)** The PAECs were untreated or treated with rhIL-17 (100 ng/ml), rhIL-1β (20 ng/ml) or rhIL-17 plus rhIL-1β for 24 h, and the expression of SLA-I **(A)** was measured *via* flow cytometry. **(C)** The PAECs were untreated or treated with rhIL-17 (100 ng/ml), rpIFN-γ (40 ng/ml) or rhIL-17 plus rpIFN-γ for 24 h, and the expression of SLA-I was measured *via* flow cytometry. The degree of SLA-I binding to PAECs was evaluated by determining the geometric mean fluorescence intensity (Gmean). Data are representative of at least three independent experiments (mean ± SEM). *$p < 0.05$, ***$p < 0.001$, determined by Student's *t*-test.

IL-4 protected porcine ECs from human antibody-mediated CDC by partially increasing claudin 5 expression (20). We previously found that TNF-α promoted porcine EC killing by decreasing occludin expression (21). Moreover, claudin 2 protected porcine ECs from human antibody-mediated CDC (21). The present study found that hIL-17 plus hTNF-α decreased the expression of four tight junction genes and increased the expression of four tight junction genes. We also found that hIL-17 plus hTNF-α obviously decreased the mRNA and protein levels of occludin. As expected, hIL-17 plus hTNF-α increased the cytotoxicity toward PAECs in human antibody-mediated CDC. In addition to occludin, whether other regulated tight junction genes contribute to IL-17 plus TNF-α-mediated cytotoxicity promotion in human antibody-mediated CDC needs to be investigated.

T-cell response is important for the cellular immune response to a xenograft (32). SLA-I is primarily responsible for $CD8^+$ T-cell activation. Previously, we found that TNF-α and IL-1β increased the expression of SLA-I in PAECs (12). In the present study, we found similar results. Interestingly, we found that hIL-17 almost completely blocked hTNF-α-mediated SLA-I upregulation.

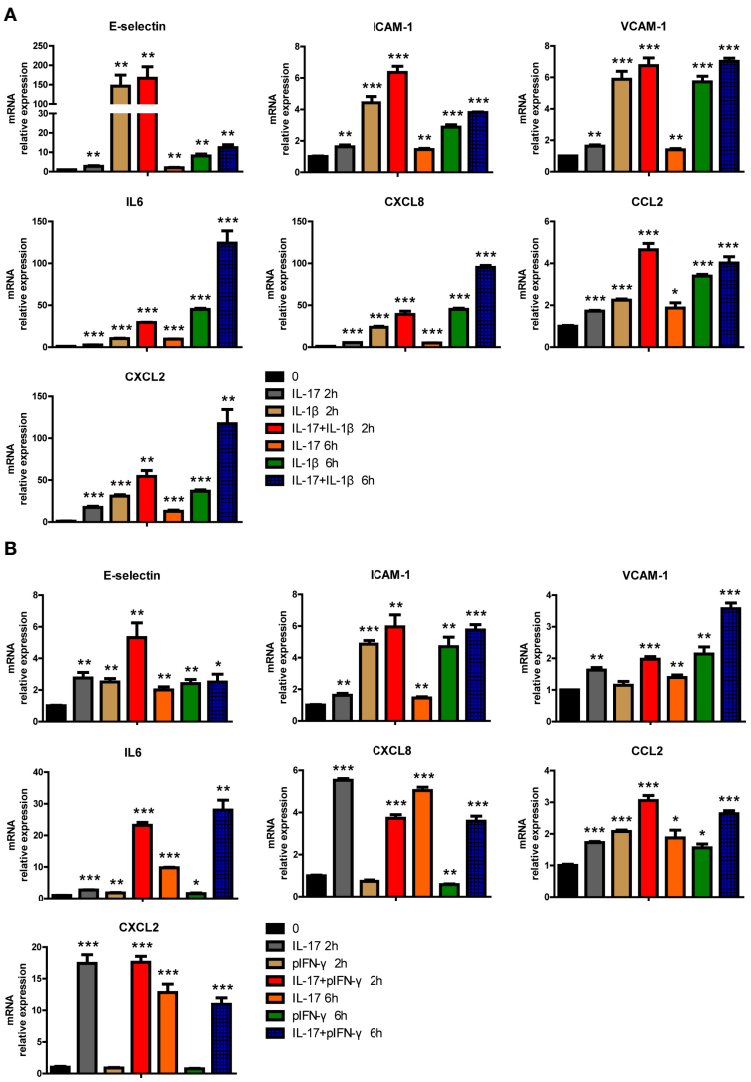

FIGURE 7 | IL-17 and IL-1β or IFN-γ additively or synergistically induced the expression of certain proinflammatory genes in porcine aortic endothelial cells (PAECs). **(A)** The PAECs were treated with rhIL-17 (50 ng/ml), rhIL-1β (10 ng/ml), or rhIL-17 plus rhIL-1β for 0, 2, or 6 h. The induction of E-selectin, VCAM-1, ICAM-1, IL-6, IL-8, MCP-1, or CXCL2 mRNA was measured using real-time PCR. **(B)** The PAECs were treated with rhIL-17 (50 ng/ml), rpIFN-γ (40 ng/ml), or rhIL-17 plus rpIFN-γ for 0, 2, or 6 h. The induction of E-selectin, VCAM-1, ICAM-1, IL-6, IL-8, MCP-1, or CXCL2 mRNA was measured using real-time PCR. Data are representative of at least three independent experiments (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, determined by Student's *t*-test.

Human IL-17 also partially inhibited hIL1β-mediated SLA-I expression. However, hIL-17 did not suppress porcine IFN-γ-induced SLA-I expression. The data suggest that hIL-17 differentially regulates TNF-α-, IL-1β-, or IFN-γ-induced SLA-I expression, and the detailed molecular mechanism needs to be investigated in the future. Human IL-17 might suppress $CD8^{+}$ T-cell-mediated cell killing in xenotransplantation. In addition to IL-17 and TNF-α, IL-17 and IL-1β or IFN-γ also had additive or synergetic effects, and whether IL-17 and IL-1β or IFN-γ also play pathological roles in xenotransplantation needs to be investigated.

Regarding the additive or synergistic effect of hIL-17 and hTNF-α, the combined inhibition of IL-17 and TNF-α has additive or synergistic effects in the therapy of certain diseases. In rheumatoid arthritis, a previous report found that the combined inhibition of IL-17 and TNF-α was effective in blocking tissue destruction associated with arthritis (16, 33). Moreover, the combined blockade of IL-17 and IL-1β showed beneficial synergistic effects to prevent joint inflammation, cartilage destruction, and bone damage in a collagen-induced arthritis mouse model (34). These studies suggest that the combined blockade of hIL-17 and hTNF-α might have superior efficacy over anti-IL-17 or anti-TNF-α blockade alone.

In conclusion, in the present study, we found that (i) hIL-17 and hTNF-α synergistically induced the expression of hundreds of genes in PAECs, (ii) hIL-17 and hTNF-α additively or synergistically induced the expression of various

proinflammatory genes and certain anti-inflammatory factors, and (iii) hIL-17 plus hTNF-α promoted immune cell migration and human antibody-mediated CDC, increased procoagulation gene expression, and inhibited anticoagulation gene expression. Further *in vivo* experiments are needed to confirm these pathological roles in xenotransplantation. Overall, coblockade of IL-17 and TNF-α might be a promising way to increase xenograft survival.

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 in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Biomedical Research Ethics Committee of the Guangdong Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HG, XC, SL, and LM designed the experiments. HG wrote the manuscript. HG, WL, PC, YZ, and MC conducted the experiments and analyzed the data. WH, LP, HS, DH, HW, ZS, and HZ helped with the experiments. WL and PC analyzed the transcriptome sequencing data. HG and XC supervised the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.857311/full#supplementary-material

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REVIEW
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Immune-Protective Formulations and Process Strategies for Improved Survival and Function of Transplanted Islets

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Type 1 diabetes (T1D) is an autoimmune disease caused by the immune system attacking and destroying insulin-producing β cells in the pancreas. Islet transplantation is becoming one of the most promising therapies for T1D patients. However, its clinical use is limited by substantial cell loss after islet infusion, closely related to immune reactions, including instant blood-mediated inflammatory responses, oxidative stress, and direct autoimmune attack. Especially the grafted islets are not only exposed to allogeneic immune rejection after transplantation but are also subjected to an autoimmune process that caused the original disease. Due to the development and convergence of expertise in biomaterials, nanotechnology, and immunology, protective strategies are being investigated to address this issue, including exploring novel immune protective agents, encapsulating islets with biomaterials, and searching for alternative implantation sites, or co-transplantation with functional cells. These methods have significantly increased the survival rate and function of the transplanted islets. However, most studies are still limited to animal experiments and need further studies. In this review, we introduced the immunological challenges for islet graft and summarized the recent developments in immune-protective strategies to improve the outcomes of islet transplantation.

Keywords: islet transplantation, immune reactions, biomaterials, islet encapsulation, cell therapy

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INTRODUCTION

Type 1 diabetes (T1D) is a chronic, immune-mediated disease. The insulin-producing β cells in the pancreas are destroyed by the autoimmune system, leading to hyperglycemia. It has been acknowledged that patients with T1D suffer from more considerable medical costs and increasing mortality and are at a high risk of developing other complications, such as chronic kidney disease, infections, osteoporosis, and cardiovascular disease (1). There is an urgent need to pursue a better therapeutic schedule and relieve the suffering of individuals with T1D patients. The main goal of T1D treatment is to maintain blood glucose at a normal range to reduce severe diabetes-associated complications. In the early 1920s, the discovery of insulin revolutionized diabetes treatment and converted a rapidly fatal disease (especially for those with T1D) to a

chronic condition. Insulin therapy could help keep patients' blood glucose within a narrow range, decreasing the risk for diabetic complications (2) and improving their overall quality of life. However, insulin-based therapy is not a perfect treatment regimen. The expensive medical cost and life-long subcutaneous insulin injections are still troubling for these patients. In addition, blood glucose monitoring, insulin dosing, diet, and exercise require strict attention. More importantly, good glycemic control is not available for all patients, and some of them even experience serious side effects of insulin therapy, including hypoglycemia and allergies.

Pancreatic β-cell replacement therapy aims to maintain normal blood glucose levels by restoring endogenous and regulated secretion of insulin and other hormones. The successful simultaneous kidney–pancreas (SPK) transplants performed in two patients with end-stage diabetic nephropathy in 1966 are effective proof of concept (3). It should be noted that pancreas transplantation is a major surgery that carries a significant risk of surgical complications and immunological rejection, most of which are related to the exocrine tissue. In contrast, islet transplantation is a less invasive alternative to transfer healthy and functional pancreatic β cells. The modern era of islet transplantation began in 1972 with the report from two laboratories demonstrating the successful reversal of diabetes in rodents (4). However, isolated islets are still susceptible to immunological rejection to some extent despite maintenance immunosuppression. After more than three decades of investigation, in 2000, the Edmonton group reported their remarkable work that 100% of patients (n=7) with labile diabetes who received islet transplantation and corticosteroid-free immunosuppression become insulin independent (5).

Indeed, the apparent effectiveness of the Edmonton protocol renewed global interest in islet transplantation as a viable T1D therapeutic option. Over time, islet transplantation has improved significantly, with numerous additional enhancements involving optimum isolation procedure, culture, securer transplant procedures, and much efficient anti-inflammatory and immunomodulatory approaches.

Although the Edmonton protocol made gradual progress, it has not entirely gotten rid of external insulin. Over time, only 10% of patients were found to be independent of external insulin over 5 years. In addition, these patients, with the treatment of islet transplantation, need systematic immunosuppressive regimens, which are associated with several side effects such as insulin resistance, nephrotoxicity, and increased risk of cancer and infections (6). Therefore, new strategies are urgently needed to avoid the lifelong use of immunosuppressive agents, improving the graft survival and secretory function.

CHALLENGES ASSOCIATED WITH ISLET TRANSPLANTATION

From the moment they are transplanted into the body, islets would be detected by the host's immune system, which will respond against them. As presented in **Figure 1**, islet-graft-confronted immune responses can be divided mainly into three types, including (1) autoimmune recurrence and alloimmunity, (2) instant blood-mediated inflammatory reaction (IBMIR), and (3) hypoxia and oxidative stress.

Alloimmunity and Autoimmune Recurrence

One of the most effective processes performed by the immune system is the response against the foreign invader, including transplanted allograft. The acute and accurate rejection response is mainly due to the dendritic cells (DCs), one type of the most powerful professional antigen-presenting cells (APCs). They can express major histocompatibility complex (MHC) class I and II antigens, that is why the DCs enable to activate both CD4+ helper and CD8+ cytotoxic T cells. In general, there are three ways to prime the T cells. In a direct way, DCs can migrate from the graft islets to the secondary lymphoid organ, present donor MHC molecules, and activate the alloreactive T cells. In a semidirect pathway, DCs and other APCs can phagocytize allogeneic cells, present the donor allogenic MHC molecules on their surface, and then activate the T cell. In an indirect way, allogenic proteins are degraded by recipient APCs, and autologous MHCs present the allogenic peptides derived from them. These allopeptide–self-MHC complexes are recognized by the T-cell receptor (7). The difference between the semidirect and indirect allorecognition is whether peptides derived from allogeneic transplantation antigens are displayed on autologous MHC class II molecules expressed on autologous APCs.

The alloreactive T cells play an essential role in allograft immunity. Migration of the activated T cells to the grafts will destroy the islets. Usually, CD8+ T cells secrete cytotoxic molecules such as perforin and granzyme B to damage the islets directly, while CD4+ T cells do not generally exhibit cytotoxic activity, but it will help boost CD8+ T cells and secrete some pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) (7). These cytokines can recruit more immune cells to reject and kill islets. They also promote macrophages to polarize into

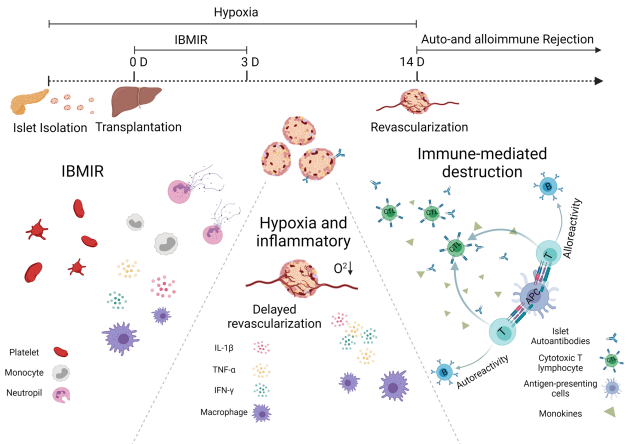

FIGURE 1 | Immunological challenges associated with islet transplantation including IBMIR, hypoxic/oxidative stress, and rejection from host immune system.

the M1 phenotype and stimulate a positive feedback loop, increasing cytokine production and killing more islets (8). Since T cells are essential for allograft rejection, current clinical immunosuppressive strategies primarily target T cells. More and more evidence suggest that B cells also play a major role in long-lasting chronic rejection of allogeneic transplantation (9). In non-human primates (NHPs), the addition of Rituximab (a monoclonal antibody that targets B cells) to anti-thymocyte globulin induction and a limited course of rapamycin successfully prolonged islets allograft survival for many years after rapamycin discontinuation (10).

T1D is a disease characterized by the destruction of insulin-secreting β cells attacked by autoimmunity. The pathogenesis might also damage newly implanted islet grafts; unlike the alloimmunity, which requires alloantigen-presenting APCs to prime T cells activation, autoimmune memory can directly reawake silent original autoreactive T cells after islet transplantation. This theory was confirmed by the fact that syngeneic islets were still largely damaged in autoimmune diabetic recipients observed in a twin-to-twin pancreas transplantation experiment (11). From another point, the precise role of autoantibodies against β-cell autoantigens, e.g., insulin-specific autoantibodies, insulinoma antigen, zinc transporter-8, and glutamic acid decarboxylase, in the pathogenesis of T1D remains unclear. However, they are the most reliable markers for assessing the autoimmune process leading to T1D. Individuals with two or more autoantibodies are more likely to develop T1D than those with only one autoantibody. Regardless of the source of β cells transplanted into a patient with T1D, autoimmune T cells would target and attack the newly implanted insulin-secreting cells. Strategies that inhibit or remove autoimmune T cells have been adopted to prevent this from happening to facilitate long-term transplanted islet function (12). Therefore, autoreactive T cells (especially CD4 and CD8 T cells) play a central role in the apoptotic β-cell destruction and could act as viable therapy intervention targets.

Both alloimmune and autoimmune responses contributed to the decrease in the graft's survival, but which one represents a more significant obstacle to the success of the islet transplantation currently remains unknown.

Instant Blood-Mediated Inflammatory Reaction

So far, intraportal islet transplantation infusion remains the leading choice for patients that need islet replacement in clinic. However, the profound islet attrition that occurs in the immediate post-transplant period blocks the success of this regimen. One primary reason for the loss of islets is termed instant blood-mediated inflammatory reaction (IBMIR). The pathomechanism of IBMIR is the thrombotic/inflammatory cascade that starts with the activation of coagulation and complement system. An *in vitro* vascular model indicated that the islets appeared to clot in 5 mins after contacting the blood (13). Rapid platelets binding onto the islet surface often cause a significant reduction of platelet in the blood and promote fibrin formation around the islet graft. In addition, those bound platelets presented upregulated expression of p-selectin and β-thromboglobulin, indicating that these platelets have been activated and this platelet-activated response could directly happen on the transplanted islets. During the first 5 min, a rapid insulin release was observed as platelets bonded to the surface of the islets. Initially, scientists believed that the complement-mediated damage mainly causes insulin secretion. However, the short occurrence time and the lack of complement activation byproducts object to this hypothesis. The most likely reason is that the activated platelets release factors such as Ca^{2+}, ATP, and ADP, which are stimulated following insulin release (14). In addition, during this process, released inflammatory cytokines also contributed to the apoptosis and necrosis of transplanted islets. In addition to causing direct islet loss, IBMIR can also promote antigen presentation, leading to an accelerated and enhanced cell-mediated immune response in the later stage of islet transplantation (15).

Different approaches have been followed to protect islet grafts from detrimental IBMIR, including inhibiting coagulation, complement activation, and leukocyte recruitment (16). Specifically, these approaches could be achieved by using soluble inhibitors, islet surface modification, and adding assistant functional cells (17, 18). The glycosaminoglycan low-molecular weight dextran sulfate (LMW-DS) can inhibit both complement and coagulation cascades (19). The LMW-DS inhibited macroscopic clotting and IBMIR in cynomolgus monkey models (20). CD39 is a critical thromboregulatory molecule expressed on the luminal surface of quiescent endothelial cells, which could limit platelet activation. Karen et al. found that when incubated with human blood, islets isolated from CD39 transgenic mice significantly delayed clotting time compared to wild-type islets (21). In addition, the surface modification of islets to avoid direct contact with blood or immune cells might be another feasible strategy. This technique could prolong the graft survival compared with bare islets in the liver of diabetic mice without doing harm to the secretory function (22).

Hypoxia, Oxidative Stress, and Inflammatory Reactions

ROS is the byproduct of oxidative phosphorylation, produced from various sources in cells, including xanthine oxidase, cytochrome 450, and mitochondria. The electron transport chain (ETC), consisting of five multi-subunit protein complexes (complexes I–V), is located in the inner mitochondrial membrane. During phosphorylation, approximately 1%–2% of O_2 reacts with electrons leaked from complex I and III, leading to partial reduction of O_2 and superoxide generation (O_2^-). Due to the high rate of O_2 consumption in mitochondria, it is believed that mitochondria are the primary source of ROS in cells. Mitochondria is not only the critical organelles for cellular metabolism but also the important O_2 sensing detector. In response to hypoxia, excessive ROS are released from complex III, which has the ability to stabilize hypoxia-inducible factor 1 alpha (HIF-1α). Hypoxia could also stimulate ROS production from complex I, which also contributes to HIF-1 stabilization (23).

In normoxia conditions, HIF-1α is oxidized (hydroxylated) by prolyl hydroxylases (PHDs) with α-ketoglutarate derived from the tricarboxylic acid (TCA) cycle, which becomes ubiquitinated, then catabolized by proteasomes. Such HIF-1α is continuously synthesized and degraded. Under hypoxia, the stabilized HIF-1α is translocated into the nucleus and dimerizes with HIF-1β and, in turn, binds to a core hypoxia response element in a wide array of genes involved in a diversity of biological processes and directly transactivates glycolytic enzyme genes. This HIF-mediated adaptation to hypoxia is important for cell survival. However, ROS are highly reactive. Excessive ROS can cause oxidative damage to lipids, proteins, and nucleic and elicits cell apoptotic cell death (24).

In the early stage of transplantation, the survival of transplanted islets is highly dependent upon oxygen supply. According to reports in the literature, islets that account for only 1% of the total pancreatic mass receive about 10%–15% of arterial blood (15), which indicates that the amount of O_2 per islet volume that is transported by hemoglobin is much larger than that of another pancreatic parenchyma. This phenomenon also suggests that islets are more dependent on high oxygen than normal cells. Thus, it is not hard to understand that islets are more vulnerable toward hypoxia than other cells. In healthy tissues in the physiological state, ROS could be neutralized by effective intracellular antioxidant systems. However, islets usually possess a weak antioxidant defense system and hold poor capacity to scavenge ROS and other free radicals, making them particularly susceptible to hypoxia and following oxidative stress (25). During the isolation procedure, enzymatic and mechanical digestion could both harm the delicate pancreatic islets. After transplantation, the process of revascularization usually requires more than 10 days, while complete vascular remodeling can take up to 3 months (26). In the meantime, islet survival mainly depends on the passive diffusion of nutrients and oxygen, which is far from enough. The islets are subjected to hypoxia throughout the process from isolation to transplantation and might cause substantial islet loss.

The extreme hypoxia environment leads to the mass necrosis of islets, triggering the innate immune response. Although the mechanism has not been fully explored, recent studies suggest Toll-like receptors (TLRs)-related pathways might play an essential role in this process (27). TRLs are a family of pattern recognition receptors that bind to endogenous ligands released by damaged cells (damage-associated molecular patterns, DAMPs), which can be released in large quantities by the mass apoptotic islets early after transplantation (28). The TLR signaling pathway will ultimately lead to the production of inflammatory cytokines through the activation of the nuclear factor-κB (NF-κB) transcription factor. In addition, the TLRs also participate in the pathogenesis of allogeneic transplant rejection. All TLRs, except TLR3, initiate myeloid differentiation primary response gene88 (MyD88)-dependent signaling, often occurring in antigen-presenting cells (APCs). In APCs, the MyD88-dependent pathway regulates cell maturation, characterized by the increased expression of CD80, CD86, MHC class II, and inflammatory cytokines, e.g., TNFα, interleukin (IL)-6, and IL-12. These events collectively contributed to enhanced T-cell stimulation and allograft rejection (29).

STRATEGIES ON HOW TRANSPLANTED ISLETS ACHIEVE IMMUNE PROTECTION

The above section describes the responses of the host immune system toward the transplanted islets. Many pieces of research have been carried out in human or non-human models to solve the problems. We outlined recent strategies for keeping the immune functioning while the grafts can evade the host immune system (**Figure 2**). Among these approaches, the use of immunosuppressive agents is trying to solve the rejection in islet transplantation by inducing immune tolerance. Encapsulation and scaffold are based on pharmaceutical science that isolates islets from various immune cells and provides them with an appropriate microenvironment. Alternatively, we could also choose an alternative immunological privileged site, anatomically isolated from immune cells or has mechanisms to suppress the immune response in their local microenvironment.

Use of Immunosuppressive-Inducing Therapeutics

Solid-organ and cellular transplantation require lifelong immunosuppressive regimes, increasing the risk of infection and malignancy. For all kinds of transplantation procedures, the balance between efficacy and toxicity must be sought. Immunosuppression includes an induction phase during the transfusion period and a maintenance phase during the entire period of islet transplantation. A further difficulty with islet transplantation is that most current immune-suppressive agents damage β cells or induce peripheral insulin resistance (30). It is hard to find an optional immunosuppressive strategy to overcome alloimmune and recurrent autoimmune reactions without harming the grafts.

The immunosuppressive strategies in the early development of clinical islet transplantation were proposed based on solid organ transplantation because the islet transplants were usually combined with kidney allografts in that period. Hence, most islet transplanted cases before the Edmonton protocol choose the immunosuppression regimen consisting of a combination of corticosteroids, azathioprine, and cyclosporine (31). They seem to be the cornerstone of the immunosuppressive agents for transplanted rejection. However, due to their nephrotoxicity, inhibition of islet function, and potential for diabetogenic consequences, they are far from ideal candidates for islet transplantation.

The Edmonton protocol in 2000 was a milestone in the process of islet transplantation. The team transplanted islets from human donors to the liver of seven patients *via* the portal vein. To avoid immune rejection to the allogenic islets, they design an effective immunosuppressive regimen including sirolimus, low dosage of tacrolimus, and daclizumab against IL-2

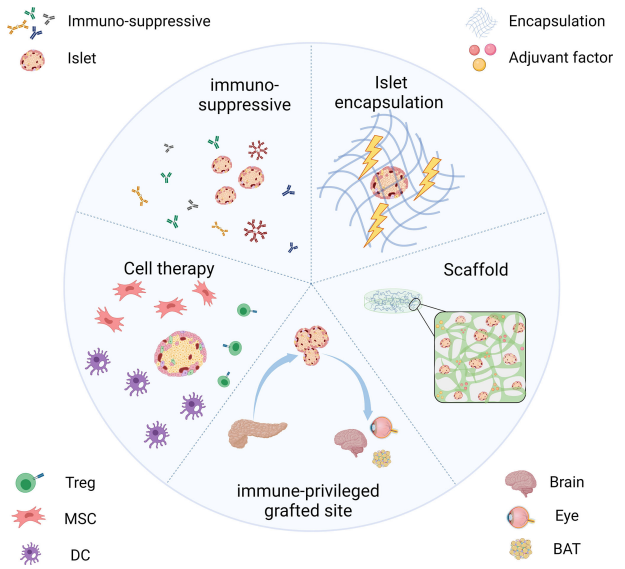

FIGURE 2 | Strategies for transplanted islets achieving immune protection include the use of immunosuppressive inducing therapeutics, islet encapsulation/scaffold to provide an immune isolate environment, choosing the immune-privileged grafted site, and co-transplantation with immunomodulatory cells.

receptors instead of traditional glucocorticoid (5). All recipients maintained normoglycemia for 3 years without extra insulin treatment; some achieved glucose homeostasis over 5 years.

The current available immunosuppressive agents can be broadly divided into two categories, the maintenance and the induction of immunosuppression. The principle of the maintenance immunosuppression is regarding lifelong inhibition of immune cells activation and proliferation, such as the calcineurin inhibitor (tacrolimus) used in the Edmonton protocol, with the clear disadvantage that most of these agents show liver/kidney toxicity and have direct toxicity to β cells. The future of achieving a permanent state of tolerance to the islet grafts without chronic immunosuppressive treatment is the pursuit of induced immunosuppression. The induced immunosuppression is adopting preemptive methods to boost the consumption of immune cells or inhibit the cell activation prior to islet transplantation. Since inhibiting T cells can decrease both humoral and cellular immunity, the induced immunosuppressive agents targeting T cells are being recognized as currently the most effective medicine (**Table 1**).

In addition, several new inhibitors have shown promising induction of immune tolerance potential. Alpha-1 antitrypsin (AAT) is a component of serum, synthesized in the liver and secreted into the blood; it is a crucial serine protease inhibitor. Recent studies have shown that AAT can suppress IFN-γ induced M1 macrophage activation/polarization by suppressing STAT1 phosphorylation and inducible nitric oxide synthase (iNOS) release. The results indicated that AAT could inhibit cytokines or dying islets induced macrophage activation, thereby improving islet survival. In addition, in recipients receiving islets and AAT, 20 of 29 reached normoglycemia, compared to only 10 of 28 in those receiving islets only, at 60 days post-transplantation (37).

Ubiquitin-editing protein A20 functions as a negative regulator of immunostimulatory factors (38). It is essential for controlling signals, including the activation of nuclear factor-κB transcription factors, which might be an ideal gene therapy candidate for islet transplantation (25). Chu et al. demonstrate that loss of A20 in B cells can cause an inflammatory syndrome with autoimmune manifestations in old mice (39). Zammit et al. designed an islet cell line that can overexpress A20 through an adenoviral vector encoding human A20 and transplanted the modified cells beneath the kidney capsule in diabetic C57BL/6 mice. The results suggested that the overexpression of A20 will reduce inflammation and prolong the survival of grafts without immunosuppression (38).

The ultimate goal of all immunosuppressive agent intervention is by preventing the allo-/auto-immunity rejection to transplanted islets to preserve their function and maintain stable glucose. Although considerable effort has been devoted to this field, a signal long-term effective therapy has not been identified. Refinement and a combination of the immunosuppressive agents mentioned above may potentially prolong the duration of glycemic control.

Islet Encapsulation

Hyperacute rejection (HAR), where host antibodies target the antigens presented on the surface of cell graft, has been proposed as the largest contributor to the immediate rejection of cellular graft (40). The principal aim of islet encapsulation is to isolate the cells from the host by a physical barrier, as presented in **Figure 3**. The encapsulating walls act as a selective barrier that prevents the transport of immune cells and large molecules (e.g., antibodies and complements) of the host immune system, which can directly or indirectly injure the grafted cells. It should be pointed out that the developed outer barrier should allow the timely bi-directional diffusion of small molecules such as oxygen,

TABLE 1 | The application of immune inducing therapeutics in islet transplantation.

Generic name	Trade name	Mechanism	Ref.
Antithymocyte globulin (ATG)	Thymoglobulin	Polyclonal antibody, profound T-cell depletion	(32)
Muromonab-CD3	Orthoclone OKT3	Anti-CD3 mAb, T-cell depletion	(33)
Alemtuzumab	CampathLemtrada	Anti-CD52 mAb, T-cell depletion	(34)
Basiliximab	Simulect	Anti-CD25 mAb IL-2 receptor antagonist	(35)
Daclizumab	Zenapax	Anti-CD25 mAb IL-2 receptor antagonist	(5)
Anti-CD154-mAb	–	Blockage of CD40/CD154 T-cell costimulation	(36)

glucose, insulin, and nutrients critical for islets between the islets and the host (41).

One of the first examples of islet encapsulation to treat diabetes was transferring human insulin-secreting tissue with a membranous bag into rats in 1933. Until the early 1950s, a series of experiments studied and compared the survival rate of allotransplanted tissue with or without cell-impermeable encapsulating membrane, and the fields of immune-isolated islet encapsulation technique were established (42). These studies confirmed that the use of the surface encapsulation technique could prevent immune cells from directly contacting and activating the antigen presentation pathway and protect islet grafts from immune attacks.

With the goal of creating immune-protected β cells, various encapsulating approaches have been developed over the past decades. Recent research on islet encapsulation has broadly divided into two directions. One is more traditional, which aims to create physical barriers by biomaterials-based encapsulation to directly reduce the contact between immune cells and islets, thus protecting islets from immune attacks. Another one seeks to optimize the grafted microenvironment of islet graft for improved islet survival and function by *in situ* release of therapeutics or oxygen.

Physical Barrier to Prevent Immune Attack

As mentioned above, it is difficult to find a suitable permeable material with precise porosity, good chemical/mechanical stability, and low immunogenicity (43). Therefore, the design and modification to chemical properties, size, and coating mode of encapsulating biomaterial have become the focus of pure physical isolation.

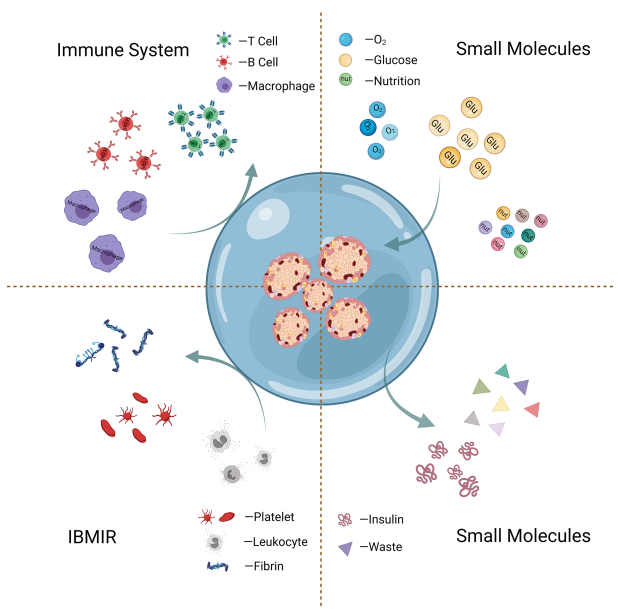

FIGURE 3 | Schematic graph of islet encapsulation that allows substance (oxygen, glucose, etc.) exchange and simultaneously acts as a physical barrier to prevent the transport of immune cells and harmful large molecules.

Microencapsulation. Microencapsulation approach is about developing immunoprotective microsized capsules coated with biomaterials for encapsulation of cells. In 1964, the cell microencapsulation technique was first described by Chang et al., and until 1980, an alginate–polylysine–polyethyleneimine microcapsule-based microencapsulation technique was first applied into islet transplantation, resulting in prolonged islet survival and normoglycemia in diabetic receivers (42). Over the next several decades, research focused on designing materials with biocompatibility for microencapsulation. As previously mentioned, the biomaterial used for islet transplantation could also induce host response and cause the formation of fibrous capsules, thus impairing the transport functions of the selective permeable encapsulating membrane. Usually, natural polymers are preferred owing to their mild properties, with alginate as the most predominantly used due to its biocompatibility, non-degradability, adjustable stiffness, and also its controllable pore size of the formed membrane to prevent cell infiltration. Alginate can form a hydrogel system *via* ionic crosslinking with a divalent cation such as Ca^{2+} and Ba^{2+}. Ca^{2+} is used more often for alginate gelling due to its non-toxicity, while Ba^{2+} could form a more robust hydrogel (44). Remarkable progress has been achieved in alginate microencapsulated islets or stem cell-derived β cells to reverse hyperglycemia. The stem-cell-derived β cell encapsulated by alginate led to the restoration of normoglycemia in immune-competent diabetic mice for 90 days (45). However, conventional alginate might induce foreign body reaction (FBR), which results in fibrotic deposition, nutrient isolation, and donor tissue necrosis. Alginate modification is a popular method of improving various aspects of islet transplantation. Vegas et al. reported that triazole-thiomorpholine-dioxide-modified alginate-encapsulating β cells derived from human pluripotent stem cells have immunosuppressive properties, allowing sustained normoglycemia glucose responsiveness for over 174 days in immune-competent diabetic C57BL/6J mice without immune suppression, even at the end of the experiment. Implants retrieved after the observation period contained viable insulin-producing cells (44).

Macroencapsulation. Despite the potential of microencapsulation, one main issue regarding alginate capsules is the difficulty to retrieve or replace them after implantation due to the complicated tissue structure and the large capsule number required for a human patient (46). Macroencapsulation devices (>1mm) may suit this need greatly. The macroencapsulation device incorporates islets into a selectively permeable membrane, which evades the immune response, enabling insulin delivery from the grafts (47). In the 1990s, Baxter Healthcare designed a planar pouch featuring a bilaminar polytetrafluorethylene membrane system named Theracyte™ device. The outer layer promotes tissue integration, where the inner membrane has a 0.4-μm pore size that possesses cell impermeable property. Many studies have used this device in diabetes research to protect transplanted islets from immune rejection. Gabr et al. found that the Theracyte capsule protected the xenogenic IPCs (human stem cells transplanted in diabetic dogs) from host immune rejection and prolonged the cell function duration, even for 18 months. After removal of the Theracyte capsules, fasting blood sugar levels of dogs quickly returned to pretransplantation readings, further confirming

the viable islet function (48). In addition, Kirk et al. transplanted the Theracyte™ device encapsulated human embryonic stem cells (hESCs) to mice subcutaneously. Their results suggested that this encapsulation device restricted direct contact between grafts and host cells, allowing further maturation of transplanted hESCs (49–51). ViaCyte Inc. created a macroencapsulation device termed Encaptra™, which has an outer plastic wave support matrix and an inner thin immune barrier layer to protect grafts. In 2017, ViaCyte launched the second trail using perforated macroencapsulation containing PEC-01 cells, in which cell survival will be improved by more optional neovascularization, but recipients in the trail will require full systemic immunosuppression (52). Skrzypek et al. developed a novel multibore system using non-degradable polyethersulfone (PES) blending with polyvinyl-pyrrolidone (PVP). This equipment showed excellent oxygen permeability over a large number of implanted human islets (6,000) within 7 days. The glucose-induced insulin secretion test further confirmed the maintenance of the endocrine function of the implanted cell (53).

In most macroencapsulation devices, the permeability will be considered, but the avoidance of islet clumping is often overlooked, increasing the oxygen diffusion distance and islet death (47). The shape and material of the device need to be modified to solve this problem. Stephens et al. designed subcutaneous injectable collagen oligomers encapsulation for islet loading. The oligomer matrices exhibited improved mechanical stability and resistance to proteolytic degradation compared with monomeric collagens (54). The glucose-stimulated insulin secretion (GSIS) curves and immunostaining results confirmed that the oligomer-based macroencapsulated islets have better cytoarchitecture and phenotype than the free islets. Their subsequent 90-day study also indicated that collagen oligomer-based biomaterials possessed strong immunoprotective properties and successfully prevented islet aggregation after transplantation (55). Interestingly, An et al. also developed a different method for the same anti-aggregation purpose (56). Inspired by the spider, this group designed a highly wettable, Ca^{2+} releasing non-porous polymer thread, which could promote the *in situ* formation of alginate hydrogel around the thread. In their short-term research, the device successfully transported rat islets into immunocompetent C57BL/6 mice and provided sufficient immunoprotection (56). The blood glucose level of diabetic recipients decreased to the normal range within 2 days, and the mice remained euglycemic until the device was retrieved. To prove the scalability and retrievability of this device, this group also performed some large animal experiments using dogs. At 1 month post-transplantation, the device could be quickly and easily retrieved by a minimally invasive laparoscopic procedure, indicating its clinical translational potential.

Nano-coating Encapsulation. The current most common site is the intraportal site in clinic (5), with the islets infusing into the hepatic microcirculation. An ideal encapsulation technique for clinic intraportal islet transplantation must therefore be suitable for intraportal delivery to the capillary bed, thus excluding macrocapsules or microcapsules due to their relatively big size. In contrast, nano-coating generates a biocompatible nanometer-sized encapsulating wall, which could ensure that the encapsulated islets remain in a small size and can be implanted into any site (57). Farooq Syed et al. coated isolated human islets with multilayer nano-encapsulation made from chitosan and poly (sodium styrene sulfonate) the reduction of glycemia was faster and quantitatively stronger in nano-encapsulation islets than in uncoated islets after transplantation under the kidney capsule of diabetic mice (58). Alginate is also used in nano-encapsulation due to its significant advantages. Zhi et al. used alternate layers of phosphorylcholine-derived polysaccharides (chitosan or chondroitin-4-sulfate) and alginate as nano-coating materials to encapsulate islets (59). In a syngeneic mouse model, no deleterious response to the coating was observed, and more importantly, the nano-encapsulated islets effectively reversed hyperglycemia. During the 1-month monitoring period, five of the seven mice retained the function of nano-encapsulated islets after allotransplantation. The results showed that the nano-scale encapsulation offers localized immune protection for implanted islets and can limit the early allograft loss.

Functionalized Encapsulation Layer to Modulate Immune Microenvironment

Pure physical encapsulation could protect the graft from the direct attacks of the recipient's immune system, but the direct immune attack is not the only problem for the transplanted islets. Hypoxia stress, inflammatory cytokine attacks, delayed revascularization, and lack of nutrients could all contribute to the death of the graft and cause severe related immune reactions. Thus, the encapsulation layer can be endowed with additional functions such as drug delivery; the local application of immunosuppressive agents can significantly reduce the dosage and directly enhance local immune tolerance. This combination of extra therapeutic drugs and resistant barriers might better protect encapsulated islets and improve overall graft survival (57).

Oxidative stress plays a crucial role in activating alloreactive and autoreactive immunity toward the engrafted islets (60). Therefore, encapsulating islets with biomaterials that possess antioxidant properties might delay or relieve immune-mediated rejections. Barra et al. generated nanothin encapsulation materials (TA-PVP) for islet protection, which is composed of tannic acid (61), a polyphenolic compound with ROS scavenging and anti-inflammatory activities, and poly(N-vinylpyrrolidone). mRNA analysis demonstrated that the TA-PVP encapsulation increases the expression of the anti-inflammatory gene Arg1 and decreases the expression of proinflammatory chemokines Ccl2, Ccl5, and Cxcl10, confirming the ability of TA-PVP to promote the anti-inflammatory innate immune phenotype and elicit localized immunosuppression. The intraperitoneal glucose tolerance test (IPGTT) curves of the TA-PVP encapsulation group were glucose responsive and similar to that of healthy mice. At the same time, islet-only grafts failed to achieve effective glycemic control, indicating that TA-PVP encapsulation successfully protected islets from immune reactions and contributed to glucose homeostasis. Bilirubin is an endogenous metabolic end-product of heme catabolism, which has the ability

of anti-inflammation, antioxidative, and immune modulation (62). The protective effect of bilirubin on T1D and islet transplantation has been widely reported (63–65). Zhao et al. developed an ϵ-polylysine-bilirubin conjugate (PLL-BR) to encapsulate the islets (66). The encapsulation matrix increased the production of superoxide dismutase (SOD) and reduced glutathione (GSH) and decreased the expression of MDA and LDH. In addition, the reduced expression of M1 markers (iNOS and CD86) and the increased expression of M2 markers (CD 206 and Arg-1) in the PLL-BR-treated group indicated that the PLL-BR could regulate macrophage polarization effectively. After transplantation of PLL-BR encapsulate islets in diabetic mice, the recipients maintained normoglycemic for 5 weeks, 2 weeks longer than the animals with untreated islets. Therefore, therapeutic-drug-included encapsulation strategies could more efficiently protect the islets from hypoxia-induced oxidative stress, especially in the early stage post-transplantation.

Adequate oxygenation of the transplanted islet remains challenging (67). *In situ* oxygen production and exogenous oxygen supply are two main methods to address the inadequate oxygenation issue. Thus, it is beneficial to combine the *in situ* oxygen generation strategy into the islet encapsulation technique. Coronel et al. developed an oxygen-generating device based on calcium peroxide, which is hydrolytically active to generate oxygen *via* the chemical reaction without enzyme. After transplantation, 100% of the recipients transplanted with the device achieve euglycemia with a mean time of 7 days, compared to the 50% of animals receiving implants only with a delayed reversal time at 14 days post-transplantation (68). *In situ* oxygen production is undoubtedly smart, but recycling waste into oxygen could be an attractive and economical alternative. Wang et al. presented an encapsulation system that could generate oxygen from their own waste product CO_2 in a self-regulated way (69). The gas–solid (CO_2–lithium peroxide) reaction that generates O_2 based on the chemical reaction between Li_2O_2 and CO_2 was utilized to make that happen, as indicated in **Figure 4**. They encapsulated 500 islet equivalents (IEQ) of rat islets and transplanted them into the dorsolateral subcutaneous space of STZ-treated diabetic C57BL6/J mice. As shown in **Figure 4**, normoglycemia was achieved in 8 of 10 inverse-breathing encapsulated-islet-treated mice and maintained for 92 days, whereas all control subjects went back to being hyperglycemic at 30 days after transplantation. On day 92, the retrieved islets maintained smooth and intact morphology, suggesting that the islet was properly preserved. This group also studied the potential of this inverse-breathing oxygen-generation technique in a larger animal model that xenotransplant rat islets into Göttingen minipigs. The islet grafts were retrieved after 1 month and examined to indicate the subrenal microenvironment with or without oxygen generation. As the result shown, most islets presented as fragmented or necrosed in the control group, revealing that the subcutaneous space is challenging for islet survival. In contrast, the inverse-breathing encapsulated islets showed a high survival rate, and most of them presented a healthy morphology after retrieval at 1 month. This result demonstrated that inverse-breathing encapsulation contributed to the generation of oxygen and improvement of anoxic microenvironment.

The islets are highly vascularized within the pancreas, which is crucial to maintain their capacity to secrete insulin quickly in response to elevated blood glucose. The isolation procedure breaks the connections between systemic circulation and the islet vasculature. It results in significant ischemic and mechanical injury, leaving islets more susceptible to post-transplant stress. Pre-vascularization of the encapsulation system has been proven to help isolated islets accommodate the transplanted site by accelerating the vascularization process. For example, Weaver et al. developed a vasculogenic polyethylene glycol (PEG) encapsulation hydrogel for islet delivery to the extrahepatic islet transplant site. This system consists of an S–S cross-linked PEG hydrogel core and a proteolytically degraded vasculogenic outer layer (67). Once transplanted, the encapsulation system could be enzyme-responsively degraded, significantly enhancing localized vascularization and re-implementing the blood supply for transplanted islets. The increased vascular density contributes to higher oxygen tension, which is beneficial for the survival and function maintenance of cells in the device. This hypothesis was proved to be correct by both whole-mount confocal and lectin perfused cross-sectional imaging after 4 weeks. Additionally, finite element analysis confirmed the increased oxygen concentration after pre-vascularization treatment, with an average central oxygen tension within the gels of 0.027 and 0.018 mM for vasculogenic PEG hydrogel and non-vasculogenic PEG hydrogel, respectively.

The inconsistent long-term efficacy hindered the translation of transplanted encapsulated islets to treat T1D in humans. The encapsulation can act as an immune barrier and deliver adjuvant drugs to solve various graft problems post-transplantation. It has been pointed out that one of the major challenges for islet encapsulation is the biocompatibility issue, which could generate a severe inflammatory response. A drug loading encapsulation system could successfully prevent early inflammation post-transplantation. For example, Maurizio et al. developed a ketoprofen-loaded islet encapsulated system to prevent early loss (70). Biodegradable microspheres containing ketoprofen were enveloped into the well-established alginate/poly-L-ornithine/alginate capsules through the layer-by-layer method. Their results indicated that this islet microsphere had high biocompatibility and the ability to reduce inflammatory reactions and pericapsular fibrotic overgrowth altogether.

Islet encapsulation has achieved leapfrog progress because of advances in material sciences, nanotechnology, and pharmaceutical sciences. In particular, the functionalized encapsulation that provides additional properties could endow isolated islets with enhanced survival and function *in vivo*, especially in the early stages of post-transplantation. Those easy-to-make biomaterials with multi-functions were very competitive in the views of clinical translation. In addition, continuous advances in materials and

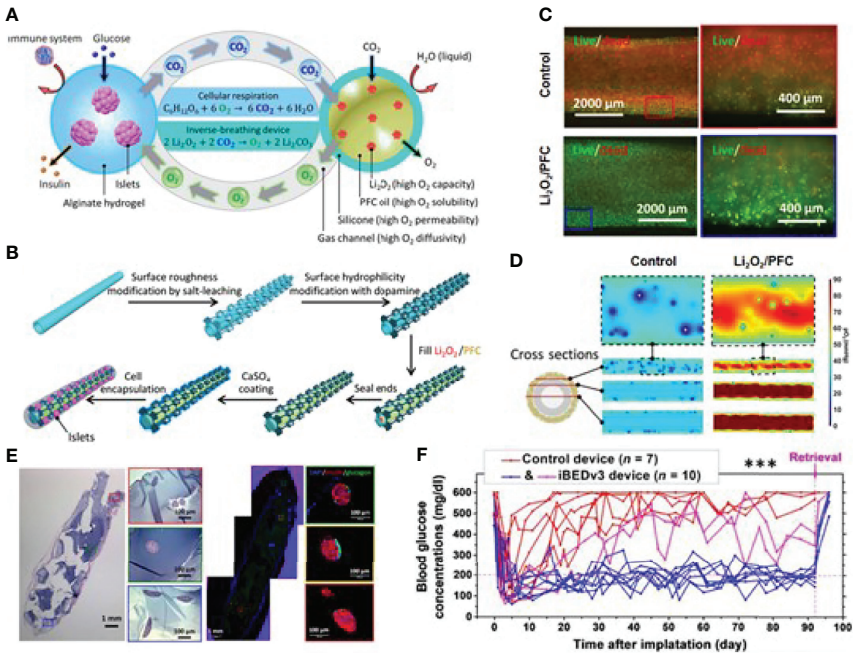

FIGURE 4 | An inverse-breathing system-based islet encapsulation. **(A)** Schematic illustration of the inverse-breathing system: CO_2 released from semipermeable alginate-hydrogel-encapsulated islets transported to PFC-encapsulated Li_2O_2 particulates, converted to O_2. **(B)** Fabrication of Li_2O_2/PFC-containing constructs. **(C)** Fluorescent microscopy images for the viability of INS-1 cells culture in a hypoxic environment with different treatments. **(D)** pO_2 distributions in three cross-sections of each design. White regions represent necrosis. **(E)** H&E and immunohistochemical staining of retrieved islet graft over 3 months. **(F)** Non-fasting blood glucose measurements after islet transplantation over 92 days. Reproduced, with permission, from (69) Copyright © 2021 The Authors.

immunology might inspire more alternatives for islet encapsulation techniques.

Scaffold Aided Islet Transplantation

The peri-islet extracellular matrix (ECM) is a scaffold of fibrillary proteins, accessory proteins, and molecules that provide structural and biological support for surrounding pancreatic islets. The peri-islet ECM provides cell anchorage and signaling that are critical for the islet's glucose responsiveness. Growing evidence shows that ECM not only acts as homeostatic support for pancreatic islets but also provides physical and immunological barriers against immune infiltration (71). Generally speaking, scaffolds engineered for islet transplant are made of biomaterials that provide mechanical support for the islets and simulate the pancreatic microenvironment. The scaffold improves the islet viability and function by promoting cell adherence and nutrient diffusion and providing ECM-mimicking support (72). In addition, scaffolds can also deliver therapeutic drugs to the implanted site. Thus, it is feasible to include immunosuppressive agents into the scaffold system and modulate early immune reactions that are directly against newly implanted islets.

The selection of biomaterials for scaffold building is crucial for successful islet transplantation. The ideal biomaterials must not cause apparent toxicity, inflammation, or even severe host response while at the same time providing enough mechanical support (73). For example, Smink et al. studied and compared the uses of three different Food and Drug Administration (FDA)-approved polymer candidates, including poly(D,L-lactide-co-e-caprolactone) (PLCL), poly(ethylene oxide terephthalate)/polybutylene terephthalate (PEOT/PBT), and polysulfone in islet transplantation (74). Culture on PEOT/PBT and polysulfone profoundly did harm to islets and induced severe tissue responses *in vivo*. PLCL was the only polymer that could sustain the cell function and survival. In a Rowett nude rat model, after transplantation of 3,500 islets in the PLCL scaffold, the hyperglycemia reversed within 3 days. Poly (lactide-coglycolide) (PLG) is also an FDA-approved biodegradable material that has been developed to build clinical drug/cell delivery (75). Biomeier et al. fabricated PLG scaffolds as a synthetic microenvironment for islets and found out the islet-PLG scaffold transplanted onto intraperitoneal fat maintained euglycemia for over 200 days in the diabetic mice (76). PLG scaffold could be modified or loaded with therapeutic drugs to achieve versatile functions to support islet graft better. Skoumal et al. designed a FasL chimeric with streptavidin-functioned PLG scaffold (77). *In vivo* data indicated that this modified PLG scaffold successfully prolonged islet graft survival from 23 days (transient rapamycin-treated) to over 200 days when the Balb/c islets in the scaffolds were transplanted into the peritoneal fat of diabetic C57BL/6 mice. Liu et al. also used a PLG scaffold for islet transplantation, and their group further explored the loading of IL-33 to achieve localized immunomodulatory in transplanted mice (78). Compared to the untreated group, the median survival time of allogeneic grafts with IL-33 loading scaffold increased

from 14 to 33 days. Some other polymers such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), polycaprolactone (PCL), and other syntheses have also been reported as scaffold materials for islet delivery. These materials are primarily used as solid scaffolds to provide mechanical stability (79).

Besides the mechanical support, it is important to endow more properties to simulate ECM function and provide a favorable microenvironment for islet graft. One direct approach is the use of ECM components, e.g., fibrin and hyaluronic acid. Fibrin is a provisional matrix protein derived from fibrinogen, extensively used as hydrogel material and sealant in clinic (80). Fibrin could bind to surface receptors like integrin and thus act as an effective cell-supportive 3D scaffold by promoting cell differentiation, proliferation, function, and survival. In particular, fibrin has a direct beneficial effect on cultured islets, including islet morphology, insulin secretion, and islet angiogenesis (81). Salama et al. subcutaneously transplanted 5,000 neonatal porcine islets (NPIs) to diabetic immune-compromised mice and studied the beneficial role of fibrin in islet transplantation (82). The grafts transplanted with fibrin achieved euglycemia between 5 and 22 weeks. In contrast, NPIs transplanted alone failed to reverse hyperglycemia under the same condition. At 22 weeks post-transplantation, mice underwent a survival nephrectomy of the graft-bearing kidneys, and then, the animals were all back to being hyperglycemic within 48 h. Fibrin could not only promote the islet survival through its biological activities but also build ECM mimicking cell/drug delivery carrier. Maillard et al. created a fibrin scaffold for islet culture by simultaneously loading perfluorodecalin, an oxygen diffusion enhancing medium (83). Perfluorodecalin was added to increase oxygen diffusion toward isolated islets and improve their function and viability as well. In this study, the scientists assessed cell apoptosis through caspase-3 activation and found out that the apoptosis of the treated group was significantly lower than that of the untreated group, indicating that fibrin matrix supplemented with perfluorodecalin loading can provide a beneficial physical and chemical environment for improved islet *in vitro*. However, fibrin clots will enable the host immune system cells to prevent infection, and many studies have indicated that fibrin can promote macrophage recruitment and cytokine production (80). This immune-mediated response might lead to chronic rejection and is not good for long-term fibrin application in islet grafts. Another concern for fibrin might be its biodegradable feature, making it unknown for the duration of fibrin to support islet *in vivo*.

The acellular scaffold is another emerging alternative for islet transplantation. Acellular scaffold usually keeps intact ECM comprising a mixture of structural and functional molecules by removing the allogeneic or xenogeneic cellular antigens from the original tissue during decellularization. The acellular scaffold can be the lair for cell protection and adhesion during the transplantation process. It is an isolated extracellular matrix from the tissues or organs of various species. It can also serve as a promoter of structural and functional repair (84). Citro et al. employed an acellular lung tissue to bio-fabricate functional islet organ, as shown in **Figure 5** (85). To provide a biocompatible multicompartment scaffold, they first pre-vascularized acellular lung matrix prior to islet seeding and obtained mature vascularized scaffold (MVS) and found out that islets could be more easily integrated into the surrounding vasculature after 7 days of culture as compared to the non-vascularized group. In response to elevated glucose levels, MVS could quickly release insulin and provide a more efficient reduction of hyperglycemia than its control counterparts. After transplantation, the MVS group can achieve euglycemia and maintain it for 30 days (**Figure 5**). A similar pre-vascularized acellular scaffold was also reported by Han et al. Their study fabricated this scaffold by coating islet-seeded fibrin hydrogen onto decellularized human umbilical arteries (86). This mini-equipment allows oxygen-rich arterial blood to flow through and provides a more islet-friendly microenvironment. When implanted, it enables restoration of normoglycemia for 90 days in the receiving diabetic nude rats. Some scientists believed that whole organ decellularization might provide a better option and generate a more pancreas-similar condition for grafted islets. Large animal studies should be conducted while exploring the use of the acellular organ for clinical application.

Scaffolds are designed to resemble the natural organs and imitate their function. An insulin-secreting bio-organ might be a perfect option. However, the choice of composite-biocompatible and scaffold architecture design still largely hurdle the further development of bio-organ scaffold in islet grafts, especially considering its long-term survival and function.

Choosing an Immune-Privileged Grafted Site

An optimal grafting site with a long-term grafting feature should be given for islet transplantation. Ideally, this site should offer venous drainage portals to enable blood glucose levels to be stabilized. In addition, the potential site should provide grafts with similar oxygen tension as the pancreas did. Some scientists pointed out that the site should also supply easy access to post-transplant islet functional and morphological monitoring (87). In terms of the immune response, grafted sites should have limited exposure to blood and immune cells to prevent inflammatory reactions. Actually, an ideal location that meets all the requirements is yet to be identified (88). We have listed some recent emerging grafted sites for islets in **Figure 6**. In the following part, we would like to introduce their own merits and demerits, with an emphasis on those that could avert immune reactions.

The liver (portal vein) is currently the preferred site for islet transplantation, constituting about 90% of clinical islet grafts. One of the major reasons to select the liver in clinical islet grafts is its procedure feasibility. Liver islet transplantation could be done through a minimally invasive approach without the need for surgery. Additionally, portal veins also enable efficient insulin delivery to avoid systemic hyperinsulinemia (88). However, the intrahepatic islet transplantation's long-term survival and efficacy are limited due to the liver-specific complications, such as islet infusion into the bloodstream will trigger IBMIR, which

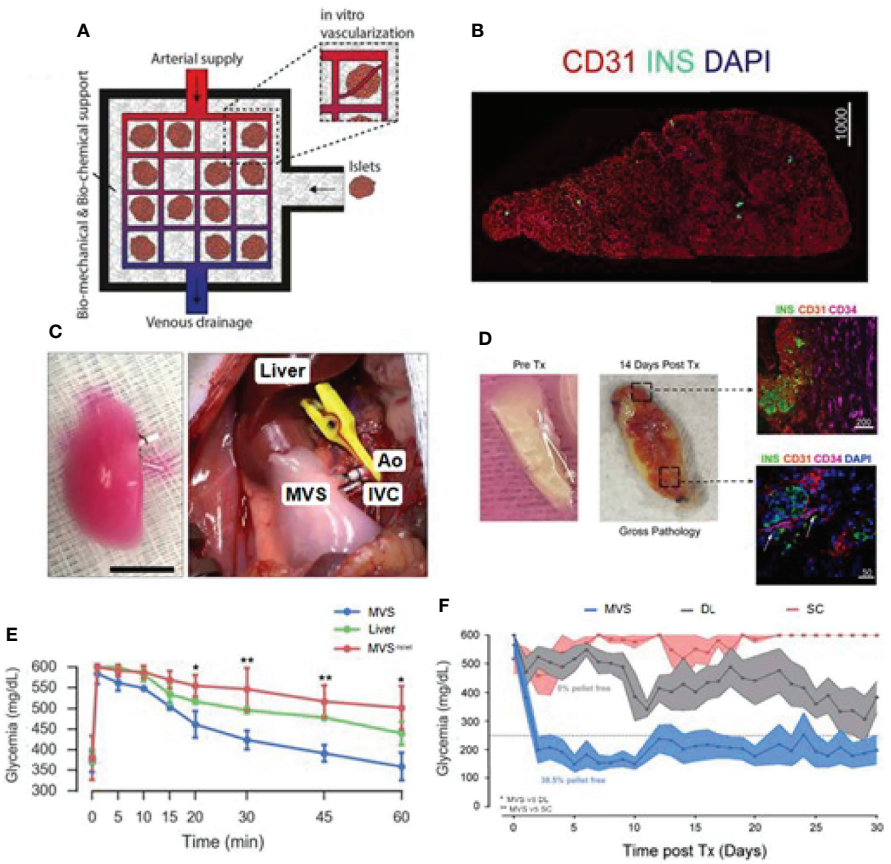

FIGURE 5 | Bioengineered mature vascularized scaffold (MVS) for islet transplantation. **(A)** Schematic illustration of the bioengineered vascularized islet organ for transplantation. (**B)** Image of a MVS' cross-section after 7 days. **(C)** Left: Images of a mature MVS for transplantation. Right: MVS transplanted into the abdominal cavity. Pulmonary artery and pulmonary veins were connected to inferior vena cava (IVC) and aorta (Ao). **(D)** MVS preTx and 14 days after transplantation. Right top image, $CD34^+$ vascular ingrowth very close to islet and human $CD31^+$ region. Right down image, murine vascular network was preliminarily established inside MVS at 14 days post-transplantation. **(E)** Blood glucose levels after intravenous glucose tolerance test in diabetic rats 1 h after vascular anastomosis. **(F)** Non-fasting blood glucose measurements after islet transplantation over 30 days. Reproduced, with permission, from (85) Copyright (2019), Elsevier Ltd.

can damage intraportal transplanted islet. Additional intraportal islet transplantation complications might include portal thrombosis and hypertension. Portal thrombosis is a life-threatening complication. Portal hypertension might raise the post-transplant bleeding risk, portal thrombosis, and the occurrence of sepsis (89).

Recently, an immunologically privileged site was constantly mentioned for islet transplants. The brain, the testis, and the anterior eye chamber are organs that inhibit the immune response and are thus known as privileged immune locations. In such sites, immune responses are largely or completely suppressed, avoiding many immunological problems that islet grafts once faced. These immune-privileged sites revisualized islet transplantation and offered a valuable occasion for expanding the survival of the allograft. However, their immune privilege mechanisms are not clearly understood. The immunological peculiarities of these sites may result from a combination of causes. For instance, the blood barrier in the retina, brain, and testis are kept immunosuppressed because of the physical cellular shield (90), while the regulatory T cells (Tregs) provide immune privilege under some circumstances as well.

The anterior chamber of the eye (ACE) has been proposed as an optimal islet implantation site. The eyes have always been considered as an immune-privileged site, which might relate to the immunosuppressive state in the anterior chamber associated with immune deviation and the tolerance related to regulatory T cells. Not only that, the ACE also provides implanted islets with an oxygen-rich milieu, directly alleviating the hypoxia of newly implanted islets. In an experimental study, allogeneic islets were transplanted into the anterior chamber of the right eye of a diabetic recipient baboon, followed by an anti-CD154 antibody (an immunosuppressor) therapy (90). Results showed that the intraocular islet allografts were retained for >400 days without subsequent immunosuppression. Furthermore, the ACE could be a novel imaging site in diabetes research for observing transplanted cells activity, since the eyes are inherently an optical apparatus (91); it allows undisturbed imaging with unparalleled penetration depth and resolution. Therefore, the status of islet graft in ACE could be monitored in real time so

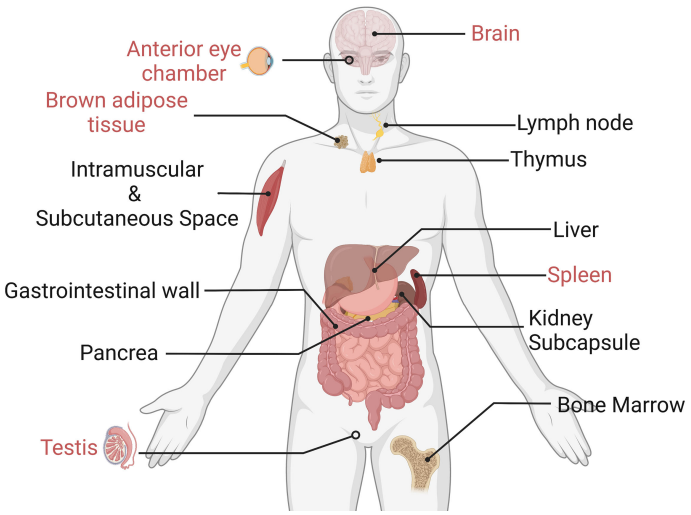

FIGURE 6 | Experimental islet transplant sites (the immune-privileged sites are marked in red). Only immune-privileged sites will be reviewed in this paper.

that appropriate immune intervention could be carried out promptly. So far, islet transplantation to the anterior chamber of the eye has been approved by FDA for clinical trial (92). However, considering the discomfort and potential effects on vision, recipients are limited to those diabetic patients with at least one eye with extensive vision loss from hand motion to no light perception. Meanwhile, some researchers are worried that transplanted islets in the ACE site are still vulnerable to autoimmunity, reminding us that immunomodulation might still be required (93).

The testis has been suggested as an immune-privileged location for islets (88). It has been reported that intratesticular islet transplantation resulted in good metabolic function that is capable of maintaining euglycemia in rats (94) and shows delayed rejection in both allograft and xenograft. Nasr et al. used a testicular islet allotransplanted model and revealed that the islet transplanted into the testis generates fewer CD8+ memory cells but induces more specific CD4+CD25+ Treg cells than that in a more conventional site (renal capsule) (95). In addition, blocking CD40/CD40L costimulation could cause intratesticular islets' immune tolerance, which is not observed in the renal subcapsular islets. These findings demonstrated that the testis is superior to inducing transplantation tolerance as an immunologically privileged site over the traditional location, e.g., renal subcapsule. While the testis may not accept a large number of islets to restore normoglycemia (96), testis islet transplantation could be conducted as a pioneering procedure to induce peripheral tolerance and protect a second site that can receive enough size of the graft.

For insulin delivery into the brain, transplanting islets into the cranial subarachnoid cavity was often exploited. The immune-privileged property and excellent nutrient supply make the subarachnoid cavity of the brain a feasible transplantation site. Most intracranial islet transplantation is operated to ameliorate cognitive impairment and peripheral metabolic dysfunctions. Bloch et al. first developed a rat model with severe dementia associated with obesity and cerebral amyloid-β angiopathy and then transplanted 100 islets into the cranial subarachnoid space (97). During the 6-month post-grafting period, the grafted islets significantly improved cognitive functions in recipients. A similar study was also conducted by Konstantin et al. (98). They transplanted the cells into the subarachnoid cavity surrounding the olfactory bulb to reverse diabetes and cognitive dysfunction. All diabetic rats achieved normoglycemia within the 2 days after receiving 3,000 IEQs and maintained it for over 2 months. Additionally, the histological results confirmed that grafted islets still preserved complete architecture after 2 months. Considering the risk of craniotomy and the difficulty of the clinical application, the brain, as an alternative transplantation site, is still under debate and needs more in-depth studies.

As an organ that is responsible for immune tolerance, the spleen is considered as immunosuppressed. The splenic T cell was reported to include suppressor T cells, which prevent dendritic cells from presenting antigens to effector T cells and suppress the proliferation of effector T cells *via* the expression of suppressive cytokines IL-35 and IL-10. Choosing the spleen as a transplant site could also reduce the islet quantity required to achieve euglycemia. Ltoh et al. studied and compared the islet numbers that are needed to achieve normal blood glucose in diabetic mice receiving islets at three different transplant sites, namely, the liver, kidney, and spleen (99). The *in vivo* data indicated that all diabetic mice gradually became normoglycemic after transplanting 50 islets into the spleen surface. The marginal number for the spleen (50) was half that for the kidney (100) and less than half that for the liver (200). The advantages of the spleen might be attributed to the physiological insulin drainage and regulation of immunity. Additionally, some researchers

believed that the spleen might also be one source of islets, with splenic cells potentially differentiating into insulin-producing cells (100). Splenic mesenchymal stem cells have also been reported to repair the damaged tissues and promote the regeneration of pancreatic islets (101).

Brown adipose tissue (BAT) could maintain thermogenesis by converting the energy into heat (102). BAT is fully vascularized, which could provide transplanted islets with sufficient oxygen and nutrient. More importantly, BAT contains rich activated M2 macrophages and Tregs, therefore displaying an overall anti-inflammatory condition. These immune-regulated cells are beneficial for islet engraftment by dampening inflammatory immune response after transplantation. Xu et al. demonstrated that islets transplanted into BAT of STZ-induced mice could restore euglycemia and maintain health glucose metabolism for over 1 year (103). After removal of islet-engrafted adipose tissue, the average blood glucose levels of diabetic mice went up to over 500 mg/dl immediately within 1 day. Kepple et al. further explored the effects of BAT islet transplantation on BAT function and immune system in recipient mice (104). Quantitative real-time PCR (qRT-PCR) data indicated no change in BAT-specific mRNA encoding Adrb3, Zic1, and the critical, thermogenic, uncoupled protein Ucp1, suggesting that the islet transplantation does not affect energy expenditure and thermogenesis of BAT. Meanwhile, islet transplants into BAT significantly delayed immune-mediated graft rejection in an allograft model. However, how exactly the human adipose tissue will affect the application of BAT as a transplantation site is still unknown. Further studies that characterize BAT mass and BAT transplantation operability should be conducted to explore BAT as a more clinically relevant graft region.

Cell Therapy

Cell therapy has emerged as a promising alternative to replace or enhance the biological function of damaged tissues using autologous or allogeneic cells. Generally speaking, islet transplantation could also be classified as one kind of cell therapy (105). Here, we focused on cell therapy that involves other cells that assist islets with their own biological functions to better survive in the transplanted site. In the following part, we will introduce some immunomodulatory cells, which have been investigated to suppress or delay immune reactions to improve the successful operative rate of islet transplantation.

Mesenchymal Stem/Stromal Cells

Mesenchymal stem/stromal cells (MSCs) are non-hematopoietic multipotent stromal cells. The ability of MSCs to secrete trophic and angiogenic factors can help early grafts rebuild vascularization after transplantation (106). Moreover, MSCs can utilize extracellular matrix as structural support and also as a bioactive molecular container. The various functions of MSC make it an attractive candidate to protect cells in islet transplantation. MSCs help the woe of grafts by targeting the major causes of post-transplantation failure—hypoxia and immune rejection. Recent studies indicated that MSCs transfer mitochondria to islets during *in vitro* co-culture, which rescue the cells from hypoxia (107). In addition, MSCs could secrete a large number of bioactive molecules that potentially affect immune and inflammatory reactions. For example, MSCs might block the differentiation of monocytes into DCs and also impair their antigen-presenting ability (108). This phenomenon may explain the profound immunosuppressive effects of MSCs on virtually any component of the immune system. Therefore, MSCs have been utilized in islet transplant to improve the overall islet survival, especially for allograft. For example, Kenyon et al. reported that MSCs and allogeneic cynomolgus monkey islets co-transplantation into the liver portal vein of the diabetic cynomolgus monkey recipient successfully prolonged allogenic islets from 24 to 81 days by increasing Tregs numbers in the periphery (109). Ishida et al. found that the MSC co-transplanted with islets intraportal transplantation inhibited the NK cells in the liver by secreting prostaglandin E2 (110) and markedly improved the islet survival.

Although MSC-assisted islet transplantation has been widely reported in a variety of animal models, most of the research mainly focused on the effect of MSCs on alloimmunity. It is still unclear whether MSCs could impede recurrent autoimmunity. In-depth knowledge of the immunomodulatory mechanisms of MSCs might help to address these concerns and promote the future application of MSCs incorporated islet transplantation in clinic.

Dendritic Cells

As part of the immune response, DCs can be critical to achieving central and peripheral tolerance. Thus, DCs could be a potential therapeutic target in the design of tolerogenic regimes (83). While mature myeloid DCs unregulated MHC class II and CD40, CD80, and CD86 costimulatory molecules, immature DCs could downregulate these markers that are active allospecific T-cell response inhibitors (111). The absence of stimulatory molecules enables immature DCs to cause particular hypo-responsiveness of antigen in T cells. Tolerogenic DCs have been shown to help allograft adoption through deletion of alloreactive T cells and activation of donor-specific regulatory T cells (Treg), and skewing the Th1/Th2 response (112). Although many studies exploit DCs as an immune target, few studies reveal its potential in islet co-transplantation. Long et al. grafted rat islets together with mouse mesenchymal stem cells (MSCs) and/or immature DCs into diabetic mice (113). The data suggested that the transplantation with either MSCs or immature DCs is better to control blood glucose levels compared to the transplantation of islets alone. In addition, co-transplantation of islets together with MSCs and immature DC obtained better results and significantly enhanced islet grafts to reverse hyperglycemia in mice with T1D. However, as for DC-assisted islet transplantation, the generation and maintenance of tolerogenic DCs remain a problem.

Regulatory T Cells

Tregs is a small and unique subset of CD4+ T cells that comprises approximately 1%–10% of normal adult peripheral blood. Tregs play a vital role in maintaining immune homeostasis and regulating inflammatory disease progressions. They suppress the inappropriate immune responses to self-antigens, such as those occurring in T1D.

Emerging evidence suggests that Tregs dysfunction might be a cause of T1D (114). Thus, those immunomodulators and cell therapies that target Tregs are considered to be of prodigious long-term potential. Yi et al. studied the effect of Tregs therapy on islet xenotransplantation (115). They transplanted neonatal porcine islets into the NOD-SCID IL2rγ−/− mice treated with or without Treg injection afterward. Treg injection treatment delayed the rejection of xenografts from 28 days (without Tregs injection) to 100 days (with Tregs injection) through a potent suppression of a predominantly CD4+ T-cell-mediated pathway. Immunohistochemical analysis indicated that no visible insulin-positive staining cells presented in the non-treated xenografts, while intact insulin-positive staining cells could be clearly observed in treated grafts (115). Co-transplantation of Tregs and islets was also studied by Naohiro et al. In their study, Tregs from C57BL/6 mice and islets from Balb/c mice were made into aggregates and loaded on agarose hydrogel with small round-bottomed wells before intraportal transplanted into C57BL/6 diabetic mice. No systemic immunosuppression was used post-transplantation. Their results suggested that Tregs in the aggregates enable six of nine transplanted grafts survival for more than 100 days (116), substantially increasing long-term allografts survival. The use of Tregs in islet transplantation is still in its infancy and needs further exploration. Tregs-related tolerance signatures need to be refined and optimized for individualized patients.

Additional cell-assisted islet transplantation could be promising. However, these cell-based strategies described above were only tested in animal models. Concerns regarding immunological compatibility, how to master multi-types of cell delivery, and general quality control and safety issues need to be addressed before moving cell therapy forward in islet transplantation application.

CONCLUSION

Islet transplantation has proven its long-term efficacy during the past decades. However, donor shortage and post-transplantation immune response limit its widespread use. With the development of stem cell and genetic modification technologies, it becomes possible to provide an unlimited number of insulin-producing cells. Thus, how to protect grafted islets without the long-term use of systemic immunosuppression has become a focused research area (117). In this paper, we introduced the immune responses against the transplanted cells and summarized recent progress in formulations and process strategies to provide immune protection for improved survival and function of transplanted islets.

A joint strategy that combines biomaterial-based encapsulation/scaffold with local immunomodulation has proven its potential in islet transplantation. This combo could protect islet graft from host immune rejection and IBMIR, and act as carriers of immunosuppression agents or assistant cells. Synthetic biomaterials are relatively easy to prepare and meet quality control requirements during mass production as safe "non-living" products. However, they have their own issues, including biocompatibility, durability, and the potential to trigger foreign body responses, which still need to be fully addressed before clinical application.

From the immunological perspective, the ideal graft sites should have restricted immune responses and ensure the lowest rate of islet loss. The so-called immune-privileged sites (e.g., brain, testis, anterior eye chamber) could meet this requirement (118). Nonetheless, these promising transplanted sites are relatively hard for surgical operation and post-transplantation monitoring. In addition, there is little experience in large animal models and human trials, making the side effects and long-term efficacy of islet transplant at the immune-privileged site rather uncertain.

Co-transplantation with immunomodulatory cells could also be a promising approach. Once transplanted, functional immune-regulating cells could act as drug reservoirs and produce cytokines and growth factors to assist co-transplanted insulin-producing cells on demand. However, problems such as maintenance of their immunoregulatory function and longevity of the cells are needed to be considered. Additionally, these cells are rare cell types and not easy to collect. In addition, further efforts should be made to ensure the stability, potency, and retention of these assisting immunomodulatory cells after the islet graft procedure.

In summary, we reviewed various immune-protective formulation and process strategies for improved survival and function of transplanted islets. The single method mentioned above cannot alleviate the dilemma faced by islet transplantation, while combining them might create long-term functional and safe cell therapies for T1D. The ultimate goal of islet transplantation is to completely cure diabetes without needing long-term immunosuppressive therapy. Although the task is challenging, success is possible.

AUTHOR CONTRIBUTIONS

Conception and design of study: QY, LK. Drafting the manuscript: YS, ZW, QW, LK, and QY. Revising the manuscript critically for important intellectual content: Y-ZZ, ZJ. All authors contributed to the article and approved the submitted version.

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Current status of xenotransplantation research and the strategies for preventing xenograft rejection

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Transplantation is often the last resort for end-stage organ failures, e.g., kidney, liver, heart, lung, and pancreas. The shortage of donor organs is the main limiting factor for successful transplantation in humans. Except living donations, other alternatives are needed, e.g., xenotransplantation of pig organs. However, immune rejection remains the major challenge to overcome in xenotransplantation. There are three different xenogeneic types of rejections, based on the responses and mechanisms involved. It includes hyperacute rejection (HAR), delayed xenograft rejection (DXR) and chronic rejection. DXR, sometimes involves acute humoral xenograft rejection (AHR) and cellular xenograft rejection (CXR), which cannot be strictly distinguished from each other in pathological process. In this review, we comprehensively discussed the mechanism of these immunological rejections and summarized the strategies for preventing them, such as generation of gene knock out donors by different genome editing tools and the use of immunosuppressive regimens. We also addressed organ-specific barriers and challenges needed to pave the way for clinical xenotransplantation. Taken together, this information will benefit the current immunological research in the field of xenotransplantation.

KEYWORDS

Xenotransplantation, hyperacute rejection, delayed xenograft rejection, chronic rejection, glucocorticoids, immunosuppressants

1 Introduction

Organ failures, which are usually a consequence of diseases, trauma, or alcohol/drug abuse, represent the top causes of mortality in most population groups (1). A recent population-based cohort study covering a 3-year period and involving 9,187 adult patients admitted at the emergency department of the Odense University Hospital, Denmark, indicated that the one-year all-cause mortality of organ failure was 29.8% (2). Another study, involving a cohort of 1,023 patients sequentially admitted at ten Scottish intensive care units, revealed a one-year overall mortality of 46.5% (3). Organ transplantation is the ideal treatment for most end-stage organ failure affecting the heart, lungs, kidneys, liver, and in certain cases, the pancreas. However, the demand of human organs for transplantation purpose exceeds by far the number of organ donations, which limits this procedure in general clinical practice. The World Health Organization (Geneva) estimates that only 10% of the worldwide need for organ transplantation is being met (4). Several countries around the world have implemented different strategies to overcome human organ shortage, including financial incentives in the United States and China (5, 6), or campaigns to increase public awareness (7). France and other European countries recently declared all citizen as donors by default in case of death or brain death unless they opted out during their lifetime. Other approaches are also explored to enable the use of animals' organs and tissues, and are referred to as xenotransplantation. In addition to addressing organ shortage issues, xenotransplants could allow for tailored transplantation and meet specific patients' needs, or offer more flexible schedules than that imposed by organ donations, usually performed on short notice (8).

The prominent pathobiological barriers to successful clinical use of xenotransplantation include the rapid activation of innate cellular responses against the graft, and at later stage, further rejection of the organ by the adaptive immune system (9, 10). As important, coagulation dysregulation and inflammation intervene in the rejection process. The long-term survival of the grafts depends on how to reduce or even avoid the occurrence of rejection, which in turn requires a deep understanding of these different rejection mechanisms. In this review, we illustrate in detail from current literatures these different mechanisms in rejection processes and the corresponding strategies to overcome them. We hope this comprehensive overview will bring new insights into current immunological research related to xenotransplantation and future directions towards its application.

2 Mechanism of xenograft rejection

2.1 Hyperacute rejection

HAR generally defines as a graft destruction occurring within 24 hours and that usually lasts for few minutes to hours. It is caused by the binding of human or non-human primate (NHP) pre-existing antibodies against graft antigens (Figure 1A) (11). Among these antibodies, most frequent IgMs and IgGs recognize galactose-α 1,3-galactose (α-Gal) residues added on glycoproteins and glycolipids by the α1,3 galactosyltransferase (*α1,3GT*) present in the genomes of non-primates and New World monkeys (platyrrhine primates living in South and Central America, including howlers, spider monkeys, and woolly monkeys) (12, 13). Humans, Old World monkeys (catarrhine primates living in African, Asian and Europe, including baboons, colobuses and mandrills), and apes lack α-Gal epitopes because their *α1,3GT* gene is affected by a loss-of-function mutation (14). In addition, about 70–90% of the antibodies produced by these species target α-Gal epitopes specifically (15). Consequently, when a pig organ is transplanted into a human or a NHP, the pre-existing anti-Gal antibodies bind to α-Gal epitopes present on the graft's vascular endothelium, and induce complement component 3b (C3b) production, complements activation (16), and formation of a membrane attacking complex (MAC). These reactions cause endothelial cells lysis, destruction of the vasculature, and ultimately, graft rejection (17, 18). The loss of endothelial vascular integrity further leads to interstitial haemorrhage, tissue ischemia, and necrosis (19, 20). Moreover, capillaries thrombotic occlusion, fibrinoid necrosis of arterial walls, and neutrophils accumulation contribute to graft failure (21). Nitric oxide species (NOS), reactive oxygen species (ROS), and other free radicals are also key components of the rejection process. The histopathological features of HAR characterize by disruption of vascular integrity, oedema, fibrin-platelet rich thrombi, and interstitial haemorrhage with widespread deposition of immunoglobulins and terminal complement products on vessel walls (21, 22).

Two ways are usually taken to prevent HAR. One consists in knocking out the *α1,3GT* gene in pigs (GTKO pigs) (23, 24), while the other relies on inhibiting complement activation by inducing the expression of human complement-regulatory proteins, i.e., hCD46, hCD55, and hCD59, on pig cells (25). Kuwaki et al. reported that the elimination of the α-Gal epitopes successfully averted HAR in baboons receiving hearts from GTKO pigs (n = 8) and increased the pig heart survival by 2–6 months (median, 78 d) (26). In a study using pig liver transplant to NHPs, genetically engineered expression of hCD55 or of hCD46 combined with GTKO was associated with a survival time of seven to nine days while the wild-type (WT) liver graft did not extend over three days (27, 28). Combination with GTKO, hCD46 or hCD55 expression reduced the early graft failure (signs of early transplantation failure within three days of transplantation) to 7% compared to 43% with GTKO organs (29).

Although these measures allow the grafts to survive beyond 24 hours, graft failure can still result from antibody recovery,

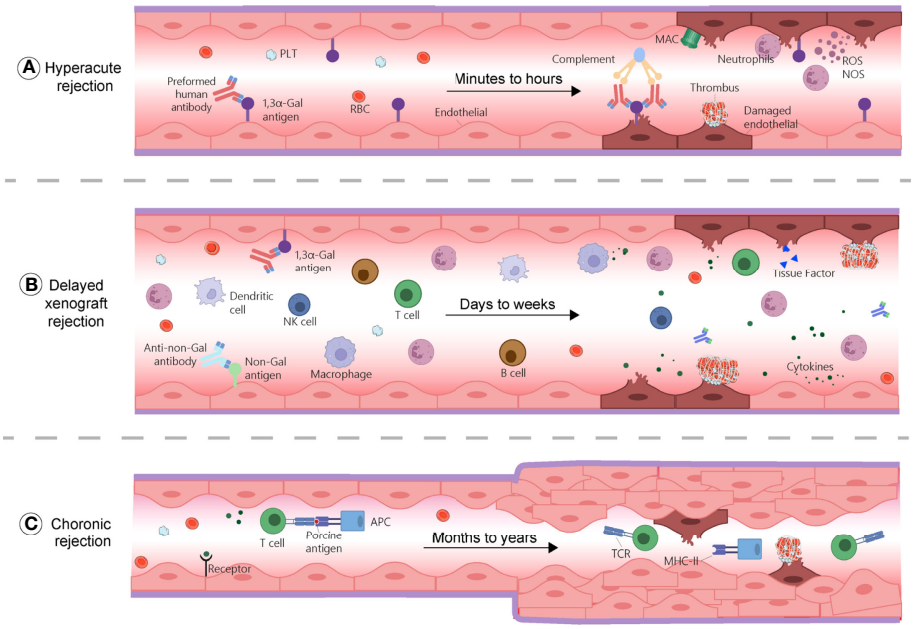

FIGURE 1
Mechanisms of rejections during xenotransplantation. **(A)** Hyperacute rejection occurs within minutes to hours and is caused by the binding of the host's pre-existing antibodies to α-Gal antigens on the graft, which results in complement activation and membrane attacking complex (MAC) formation. This reaction causes endothelial cells lysis, fibrinoid occlusion, and vasculature destruction. Neutrophils, through the production of ROS and NOS also contribute to this process. **(B)** Delayed xenograft rejection (DXR) occurs within days to weeks and include acute humoral xenograft rejection (AHXR), cellular xenograft rejection, and coagulation dysregulation. AHXR is antibody-mediated and involve non-Gal antibodies and α-Gal antibodies reactivity against non-Gal epitopes and α-Gal of the graft. Various innate and adaptive immune cells, proinflammatory cytokines, and coagulation dysregulation contribute to rejection, resulting in massive deposition of immunoglobins, fibrin, endothelial cell lysis, and interstitial bleeding. **(C)** Chronic rejection occurs within months to years. Xenoantigens are surveyed by host APCs and presented to T cells, leading to their activation and triggering inflammatory cascades, characterized by thrombotic microangiopathy, proliferation of the graft vascular endothelial cells, vessel narrowing, and interstitial fibrosis. APC, antigen presenting cell; MAC, membrane attacking complex; MHC-II, major histocompatibility complex class II; NK cell, natural killer cell; NOS, nitric oxide species; PLT, platelet; RBC, red blood cells; ROS, reactive oxygen species; TCR, T cell receptor.

through a mechanism termed acute humoral xenograft rejection (AHXR), acute vascular rejection, or delayed xenograft rejection (DXR), as explained below (20).

2.2 Delayed xenograft rejection

Delayed xenograft rejection refers to post-HAR and is also called acute humoral xenograft rejection from a mechanistic prospect, or acute vascular xenograft rejection (AVXR) from a pathophysiological prospect. AHXR or AVXR define xenograft injuries occurring within the vasculature and involving antibodies, while complements play a minor role during this type of rejection (21). In other articles, DXR may refer to antibody- and complement-independent cellular xenograft rejection (CXR) (30). Although there are clear differences between the mechanisms underlying these different terms, these are often used interchangeably and there is no international standard. Some efforts should be considered to achieve internationally recognized definitions and descriptions of these different concepts. Below, we discuss the concepts of AHXR and CXR in separate sections.

2.2.1 Acute humoral xenograft rejection

Provided that a xenograft does not fail due to HAR, a second step consist in overcoming AHXR, which causes immunological destruction within a few days to a few weeks (Figure 1B) (14). The histological characteristics of AHXR are focal ischemia and diffuse intravascular coagulation mediated by both humoral and cellar immune responses provoking endothelial cell activation and exaggerated inflammation (31, 32). Lin et al. reported that anti-Gal antibodies removal from the blood of baboons prevents AHXR of porcine organs transgenic for human decay-accelerating factor and CD59, demonstrating that Gal-specific antibodies were implicated in AHXR, in addition to HAR (33). However, antibody-meditated rejection still occurred during transplantation of GTKO pigs' kidneys or hearts into NHPs, and eventually led to graft failure over the course of several days (34, 35). These results suggest that non-Gal antigens also contribute to AHXR (34, 35).The presence in

the recipient of pre-existing antibodies against non-Gal epitopes, such as carbohydrate N-glycolyl neuraminic sialic acid (Neu5Gc), glycan SDa, defining the blood group of the same name, was also implicated in rejection (Figure 2) (36–38). Zhu et al. identified Neu5Gc as a crucial non-Gal xenoantigen in 2002 (36), while the SDa blood group was discovered in 1967 (39). Neu5Ac is hydroxylated to produce Neu5Gc by the cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) present in pigs and other animals, but not in humans (40). The gene encoding this enzyme has been inactivated during primate evolution (41). Double knockout (DKO) of the genes responsible for Gal and NeuGc synthesis in mouse reduced significantly xenoreactive antibody-mediated complement-dependent cytotoxicity of human sera towards mouse tissues, compared to wild-type mouse tissues. The gene encoding the Beta-1,4-N-acetyl-galactosaminyltransferase 2 (β4GALNT2) is responsible for the SDa positive blood group. Its inactivation can significantly diminish porcine xenoantigenicity and reduce the effects of human and NHP non-Gal antibodies (42). Therefore, Neu5Gc and SDa represent key targets for clinical xenotransplantation. Other non-Gal antigens such as Gabarapl1 (GABA type A receptor-associated protein like1), and COX-2 can also induce antibody-mediated rejection (43). In addition, shared common epitopes of human leucocyte antigen (HLA) and swine leucocyte antigen class I (SLA-I) could lead to cross-reactivity between human and porcine (44). Some studies showed that human CD8 +T cells were capable of recognizing SLA-I and elicited immune responses and anti-HLA class II antibodies in patients could cross-react with SLA-II (45–48). Immunoengineering of the vascular endothelium to silence SLA expression might be feasible to reduce the immunogenicity (49–52).

The binding IgM and IgG to non-Gal antigens triggers the onset of AHXR by activating the complement cascade, which is a key actor of antibody-mediated damages (53). Besides, humoral responses against endothelial epitopes can also cause activation and damage of the vascular endothelium through other mechanisms, for example antibody-dependent cell-mediated cytotoxicity (ADCC) and inflammation. Further, neutrophils may activate porcine endothelial cells (pECs) (54). Natural killer (NK) cells (55) and macrophages (56) are also agents of AHXR, but the exact pathogenesis involving these cells remains unclear. The histological characteristics of AHXR include extensive interstitial bleeding, infarction, necrosis, thrombosis, neutrophil infiltration, and massive deposition of immunoglobins, complements, fibrin and platelets (34). These manifestations are similar to those found in HAR (14).

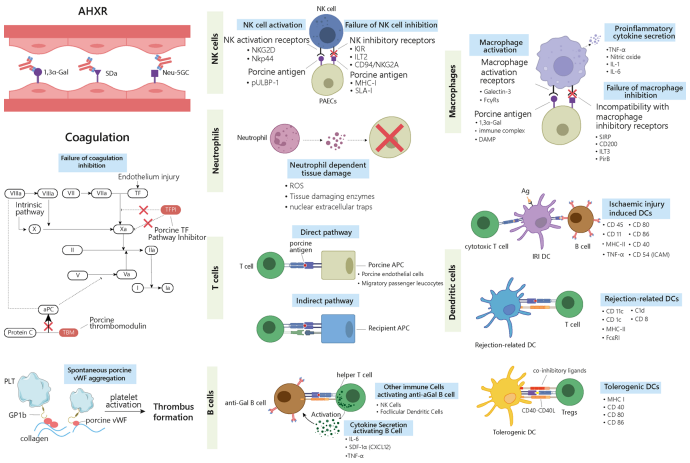

FIGURE 2
Delayed xenograft rejection: AHXR, cellular xenograft rejection and coagulation dysregulation. The antibodies involved in AHXR are mainly directed against 1,3α-Gal epitopes and non-Gal epitopes such as Neu-5GC and SDa blood group. During cellular responses, ligands on the graft cells activate recipients' NK cells and macrophages *via* different activating receptors. Meanwhile, graft cells fail to deliver inhibitory signals to the activated cells and enhance their proinflammatory and cytotoxic properties. Neutrophils can generate ROS, tissue-digesting enzyme, and NETs upon activation by graft cells, which causes tissue damage. Three types of DCs are involved in graft rejection. IRI-related DCs activate both cytotoxic T cells and B cells, thus triggering both cell- and antibody-mediated rejection. Rejection-related DCs promote acute and chronic rejection by activating T cells. Tolerogenic DCs suppress immune rejection by inducing Tregs. The different surface markers characterizing the different immune cells are listed in the right-hand side of the cells. T cells are involved in rejection through both direct and indirect pathways. In the direct pathway, porcine APCs present antigens and activate host's T cells. In the indirect pathway, the graft antigens are presented by the host's APCs. B cells are activated by T helper cells and secreted cytokines. Antibodies released by activated B cells that have differentiated into plasma cells contribute to graft rejection. AHXR and cellular xenograft rejection are both accompanied with coagulation dysregulation, where porcine TFPI cannot fully inhibit factor Xa and fails to inactivate TF. Porcine TBM also fails to regulate protein C. Porcine vWF can aggregate spontaneously and activate the host's platelets through GP1b receptors. Altogether these reactions lead to the formation of thrombus in the graft vessels. AHXR, acute humoral xenograft rejection; Ag, antigen; aPC, activate protein C; DC, dendritic cell; IRI: ischemia-reperfusion injury; NET, nuclear extracellular traps; TBM, thrombomodulin; TF, tissue factor; Tregs, T regulatory cells; vWF, von Willebrand Factor.

Genome editing tools, such as zinc-finger nucleases, transcription activator-like effector nucleases (TALEN), and CRISPR (clustered, regularly interspaced, short palindromic repeats)/Cas9 (CRISPR-associated protein 9), have paved the way for significant breakthroughs in dealing with AXHR by creating DKO (*GGTA1/CMAH* or *GGTA1/B4GALNT2*) pigs, or triple knockout (TKO, *GGTA1/CMAH/B4GALNT2*) pigs, which had been first generated in the USA (57–60). Cells from *GGTA1/CMAH* pigs were associated with reduced affinity of the human antibodies compared with cells from GTKO pigs (59). Moreover, the absence of Neu5Gc and Gal epitopes from murine xenogeneic cells has been proven to dampen the immune reaction provoked by the pre-formed antibodies contained in human serum (61). These data suggest that the deletion of the Neu5Gc epitopes could help increase the survival time of xenografts. *In vitro* evidence showed that the reactivity of pre-existing antibodies against the xenografts could be reduced by inactivating the *B4GalNT2* gene (60). This result indicates that TKO pig organs can promote major development for transplantation into human compared with GTKO and DKO xenografts, but still TKO pig organs were not sufficient to achieve prolonged graft survival, limiting by complement-mediated coagulopathies (62). It should also be noted that a fourth xenoantigen seems to be exposed when the Neu5Gc epitopes are absent, and that baboons and other Old World monkeys are more likely to reject this fourth xenoantigen than humans are (63). Therefore, TKO pig heart or kidney transplantation in baboons may appear more problematic than they would be in a clinical trial involving humans. Due to the cross-reactivity between HLA-specific antibodies and SLA, modifying SLA genes may help further reduce the human immunoglobulin binding against pig cells. Two inactivating mutational approaches targeting either the light β-chain or a conserved region in the heavy α-chain of SLA-I have been successfully utilized in the pig genome to generate pigs with no or limited expression of SLA (45). PBMC from these pigs induced almost no proliferation of purified human CD8+ T cells (45). However, complete removal of SLA in pig genomes may not be an ideal solution due to logistical, immunological, and infectious consequences of SLA deletion (64). Instead, for highly HLA sensitive recipients, an ideal organ-source pig might be one with site-specific mutations to eliminate cross-reacting antibody binding.

2.2.2 Cellular xenograft rejection (acute cellular rejection)

If both HAR and AHXR are overcome, but immunosuppressive therapy is insufficient, CXR may occur and lead to graft rejection within days or weeks following transplantation (65, 66). CXR can be mediated by the innate and/or the adaptive immune system, and may involve NK cells, macrophages, neutrophils, dendritic cells (DCs), T cells, and B cells (Figure 2).

2.2.2.1 NK cells

NK cells mediate xenograft rejection by direct NK cell cytotoxicity (NKCC) or ADCC. Upon direct contact with the target cells, NK cells deploy cytotoxic functions by engaging a series of activating and inhibitory receptors and ligands (53). Recent research has revealed a mechanism whereby NK cells adhere to and transmigrate through the porcine endothelium by interacting with an as yet to define porcine ligand, *via* CD49, integrins including CD11a/CD18 and CD11b/CD18, and CD99 (67). NKG2D and NKp44 are activating receptors that can bind porcine pULBP-1 and an unidentified ligand, respectively (68). Their engagement initiates the release of lytic granules containing cytotoxic proteins such as perforin and serine esterase, which lead to the lysis of the donor's endothelial cells (69). Inhibitory receptors such as killer-cell immunoglobulin receptors (KIRs), immunoglobulin-like transcript 2 (ILT2), and CD94/NKG2A heterodimers mainly recognize MHC-I molecules (70, 71). Low expression or absence of MHC-I molecules on porcine cells leads to reduced inhibitory signals transduced to the NK cells, which triggers their activation and subsequent lysis of the porcine cells (72, 73). Stable expression of transgenic human leukocyte antigen (HLA)-Cw3 and/or G, and/or E, on porcine cells could protect the xenograft from human NK cytotoxicity (74).

Besides direct cytotoxicity, NK cells can also employ complement-independent ADCC to destroy the graft (75). Preformed natural antibodies against α-Gal carbohydrates or Neu5Gc can bind to the pECs with their Fab portion. Fc receptors (FcRs), including CD16 (FcγRIIIa) on NK cells, recognize the Fc portion of the antibodies, triggering a signalling cascade that causes degranulation (76). In addition, CD16 recognizes induced antibodies against SLA-I, which can mediate ADCC (77). Furthermore, NK cells can promote the production of non-Gal antibodies against the graft in a T-independent manner, by interacting with splenic marginal zone B cells *via* CD40/CD154 interaction (78).

The role of NK cells in CXR needs further elucidation to prolong xenotransplant survival. To date, most knowledge comes from *in vitro* studies and animal models. Future *in vivo* studies on pig-to-NHP transplants are needed to clarify the role of NK cells in xenograft rejection.

2.2.2.2 Macrophages

Macrophages carry out diverse functions, ranging from phagocytosis, cytokine production, antigen presentation, to tissue repair. Like for other innate immune cells, Toll-like receptors on the surface of the macrophages can recognize nonself molecules such as danger-associated molecular patterns (DAMPs) arising from injured xenogeneic cells (79–82), pathogen-associated molecular patterns (PAMPs), polysaccharides, and polynucleotides (83). Under the synergistic effect of Toll-like receptors activation and interferon γ (IFN-γ), macrophages are licensed to process and

present xenoantigens, promote the differentiation of pro-inflammatory T helper 1 (Th1) and T helper 17 (Th17) by producing interleukin-12 (IL-12), and exert direct cytotoxicity by producing proinflammatory cytokines such as tumour necrosis factor α (TNFα), IL-1, IL-6, and nitric oxide (84, 85). Interspecies incompatibility of CD47 was also reported to contribute significantly to macrophage-mediated rejection of xenogeneic cells. That is, in a human macrophage-like cell line, porcine CD47 does not stimulate the inhibitory receptor signal-regulatory protein α (SIRPα), while soluble human CD47-Fc fusion protein does, which inhibits porcine cell phagocytosis (86). Human macrophages were found to phagocytose porcine red blood cells independently of the presence of antibodies or complement activation, even in setups where the α-Gal epitopes were absent from the porcine cells (87).

In CXR, macrophages mainly act by promoting T-cells mediated rejection (88–90). Feng et al. used IL-10/Fc to treat mice before or after transplantation of a pancreatic islet xenograft, and found that the T cell effector functions were inhibited, with reduced IFN-γ and IL-4 expression probably due to the inhibition of IL-12 production by macrophages (91). In whole organ xenografts, Lin et al. showed that the macrophages might be the cause of rejections occurring within 3–6 days. In their study, hamster hearts were transplanted into genetically engineered T cell-deficient rats depleted or not of NK cells, which demonstrated that in absent of T and NK cells the graft was still rejected. The spleen of the recipient and the rejected organs were predominantly infiltrated by macrophages (92). Since abundant evidence shows that macrophages play a role in xenograft rejection, the regulation of their activity might enhance the survival of future xenografts.

2.2.2.3 Neutrophils

There are at least three mechanisms whereby activated neutrophils can induce tissue damage: (i) ROS generation; (ii) release of tissue-digesting enzymes; and (iii) nuclear extracellular traps (NETs) formation (93).

Human neutrophils can directly recognize pECs, and subsequently upregulate adhesion molecules and render porcine cells more vulnerable by exposing them to NK cell-mediated cell lysis (94). Specific recognition pathways were thought to be responsible for the adhesion of human neutrophils to porcine endothelium, as under flow conditions, adhesion occurred independently of the presence of α-Gal or ICAM-1 (95). Mohanna et al. demonstrated that neutrophils can be activated directly by porcine aortic endothelial cells, which subsequently causes a transient rise of calcium flux triggering the production of reactive oxygen metabolites and inflammatory cytokines (54). Cardozo et al. further demonstrated that the adhesion of human neutrophils to pECs can be facilitated by a soluble chemotactic factor produced by pECs (96). Activated neutrophils also exert cellular damages by generating superoxides *via* NADPH oxidase activity (97).

Apart from ROS generation, leukocyte proteases have been implicated in neutrophil-mediated graft tissue damage, through disruption of endothelial cell junctional complexes (98). For instance, after acute ischemia/reperfusion insults in liver, neutrophil elastase (NE) breaks down graft's homeostatic barriers by degrading the extracellular matrix (ECM) components, including collagen, elastin, and fibronectin (99).

"NETosis", a program for production of neutrophil extracellular traps (NETs), is a unique process whereby neutrophils induce inflammation and cell death in porcine grafts. NETs are mainly composed of antibacterial peptides, histones, and serine proteases that accumulate in the lung in both experimental and clinical primary graft dysfunction (PGD). Disruption of NETs with DNase I reduces lung injury (100).

2.2.2.4 Dendritic cells

The most efficient antigen presenting cells (APCs) in activating T cells and initiating immune tolerance are the DCs. Based on different surface markers, DCs are generally divided into three subsets: (i) DCs involved in ischemia-reperfusion injury (IRI) and expressing C1d, CD8α, CD11c, CD40, CD45, CD54 (ICAM), CD80, CD86, MHC-II, and TNFα, but are negative for CD4 and CD205 (101); when activated by antigens released during ischemia-reperfusion, these DCs can trigger both cellular and antibody-mediated rejection, resulting in harmful antibodies secretion by activated B cells and killing of donor cells by cytotoxic T cells (102); (ii) rejection-related DCs, promoting acute and chronic rejection *via* different interactions with T cells and are characterized by the expression of CD11c, MHC-II, CD1c and FcεRI (103); (iii) tolerogenic DCs, suppressing the rejection process by dampening the T cell effector functions and promoting T regulatory cells (Tregs) activity; these DCs express significantly lower levels of MHC, T cell co-stimulatory molecules, such as CD40, and CD80/86, and inhibitory ligands, such as programmed death ligand-1 (PD-L1) and death-inducing ligands, reflecting their non-phagocytic profile (104, 105). In response to specific signals such as DAMPs, host DCs can acquire a rejection-related phenotype, which can further evolve towards a tolerogenic phenotype upon treatment with rapamycin, IL-10, vitamin D, or low-dose granulocyte-macrophage colony-stimulation factor (GM-CSF). Tolerogenic DCs reduce CD4+ T cell activation and impair CD8+ T cell functions, which helps suppressing graft rejection (102). Manna et al. reported that DCs activation by the porcine aortic endothelial cells could be blocked by a pre-treatment of the DCs with antibodies specific for the human leukocyte function-associated antigen-1 or CD54 (106). However, the exact mechanisms underlying DCs participation to the rejection process still need to be clarified in order to develop anti-rejection drugs.

2.2.2.5 T cells

The role of T cells in cellular rejection during pig-to-baboon xenotransplantation has been demonstrated, although the relevant studies were only few (107, 108). Upon xenotransplantation, T cells are activated by both direct and indirect pathways (109). In the direct pathway, porcine APCs expressing CD80/86 constitutively, such as pECs and migratory passenger leukocytes, can directly prime primate T cells (110). Simultaneously, primate TCRs interact with SLA-I/II peptide complexes, resulting in T cell-mediated cytotoxicity against the porcine vascular endothelium. In the indirect pathway, T cell activation occurs through the presentation of porcine peptides by the hosts' APCs. T cell activation requires antigen recognition through the TCR coupled with costimulatory signals (111), involving CD40-CD154 and/or CD80/CD86-CD28 interactions (112). Different drugs targeting these costimulatory pathways could be administrated in xenotransplantation, as for example anti-CD40 mAb and CTLA4Ig, as discussed below. In addition, abrogation of SLA-I expression has been proved to silence T-cell and NK cell-mediated cell lysis (113, 114).

2.2.2.6 B cells

One percent of total circulating IgGs, and 1–4% of total IgM in the human serum are directed against α-Gal epitopes (115). Previous studies established that the cells producing anti-Gal antibodies reside mainly in the spleen, and to a lesser degree in lymph nodes and bone marrow (116). This location matches that of a recently described splenic B cell subtype characterized by a Mac1+ B1b-like phenotype (117). Antibody production results from the interaction between B cells and other immune cells, including T cells, NK cells, and follicular DCs. Immunization of α1,3GT KO mice with pig cell membranes induces clonal expansion of anti-Gal B cells that can present antigen to T helper lymphocytes *via* MHC-II and provide CD40 co-stimulation, causing cytokine production by the activated CD4+ T cells. This process provides activated B cells with the helper signals necessary to their proliferation and maturation in germinal centres, resulting in production of high-affinity anti-Gal antibodies (118). Another study suggested that marginal zone B cells can produce xenoantibodies after receiving help from NK cells, independently of T cell help (78). Moreover, the follicular DCs, expressing the complement receptors 1 and 2, can activate α-Gal-reactive B cells by presenting α-Gal immune complexes (119).

As B cells are the main sources of elicited anti-porcine antibodies, they represent an important target to overcome AHXR. Efficient depletion of circulating and secondary lymphoid organ-resident B cells by anti-CD20 antibody at the time of transplant prevents anti-pig humoral responses and resulting graft injury, and significantly delays or prevents the systemic dysregulation of the coagulation pathway and thrombotic microangiopathy (120, 121). Zhao et al. reported that anti-high mobility group box protein 1 (HMGB1)-neutralizing antibody prolonged xenograft survival, and dampened tissue damage and immune cell infiltration by suppressing xenoreactive B cell responses (122).

2.2.3 Coagulation dysregulation in DXR

Coagulation dysregulation was first described by Ierino in the late 1990s. Both AHXR and cellular xenograft rejection are accompanied with coagulation dysregulation, which results in the development of thrombotic microangiopathy in the graft. Antibody-mediated and cellular rejections cause endothelium injury, exposing tissue factor (TF) and collagen. The binding of TF to activated factor VII (FVIIa) initiates thrombin generation, converting fibrinogen into fibrin. Simultaneously, sub-endothelial collagen triggers the accumulation and activation of platelets (123). Importantly, this process is enhanced by molecular incompatibilities between the primate and porcine coagulation homeostatic systems. The porcine tissue factor pathway inhibitor (TFPI) cannot fully inhibit the factor Xa in primates and fails to inactivate TF (124). In addition, the porcine thrombomodulin (TBM) is unable to regulate the primate thrombin, and thus, fails to activate the protein C (Figure 2) (125). Another incompatibility lies between the primate platelet lycoprotein 1b (GP1b) and the porcine von Willebrand Factor (pvWF). pvWF can spontaneously aggregate without shear stress and activate primate platelets through the GP1b receptor (126). The subsequent graft vessel thrombosis caused by fibrin deposition and platelet aggregation eventually leads to ischemic injury (Figure 2) (26, 127). Approaches to tackle coagulation dysregulation include the transgenic expression of human complement proteins (hCRP) and coagulation proteins such as human TBM by the donor porcine organs (128).

2.3 Chronic rejection

Chronic rejection usually occurs several months to years after organ transplantation. It has similar histopathological characteristics to those found in allotransplantation and are mainly related to thrombotic microangiopathy, characterized by the proliferation of graft vascular endothelial cells, vessel narrowing, interstitial fibrosis, which ultimately, result in graft failure (Figure 1C) (129). Since there are only few long-term survivors to xenotransplantation, the mechanism of chronic rejection has not been sufficiently documented, but it is almost certainly related to long-term, low-amplitude immune responses.

Current research indicates that chronic rejection involves both immune and non-immune factors. Molecular incompatibilities between the porcine and the NHP coagulation factors may play a vital role (130). Mohiuddi *et al.* reported that gene-editing of pig heart (*GTKOhTg.hCD46.hTBM*), alongside with anti-CD40 monoclonal antibody treatment, allowed for successful survival of a graft for 236 days in baboons. This result indicates a crucial role of

human TBM expression combined with anti-CD40 treatment not only for the long-term survival of the graft, but also to avoid thrombotic microangiopathy and other coagulation-related problems (131). Another study demonstrated that transgenic expression of human CD39 (a major vascular nucleotidase that converts ATP and ADP into AMP, further degraded into anti-thrombotic and anti-inflammatory adenosine) in mouse significantly prevents thrombotic events in the heart graft and improves the duration of graft survival from three days in WT mice, to six days in the transgenic mice (132).

Another study by Kim et al. achieved a long-term survival of 499 days with pig-to-rhesus macaque renal xenografts by depleting CD4+ T cells (46), indicating that these cells are responsible for chronic rejection. Similar to allotransplantation, the host's MHC class II molecules recognize porcine xenoantigens and present them to the host's CD4+ T cells, leading to their activation (109). Yet, the mechanisms involving the CD4+ T cells in chronic rejection are poorly understood. Besides, a sustained inflammatory response is still a key challenge to achieve successful grafts. Future studies should explore the roles of inflammatory cytokines such as IL-6, TNF-α, IL-17, and their inhibitors to uncover therapeutic targets (133, 134).

3 The prevention of xenotransplantation rejection

Since 2009, porcine models with new genetic modifications have been constantly implemented to improve molecular compatibilities. As gene editing techniques such as zinc finger nucleases, TALEN, and CRIPSR/Cas9 genome editing system improve, the production of multiple-gene edited pigs has become easier and faster (135). This section of the review will focus on the mechanisms and usage of common immunosuppressants in xenotransplantation area (Figure 3).

Immunosuppressive agents are used commonly in treatments for transplantation rejection. Conventional immunosuppressive therapy, for example, corticosteroids, tacrolimus, and cyclophosphamide (136–138), when used at high dosages, may delay graft failure. In non-human concordant models for kidney or liver xenografts, long-term survival can be achieved with conventional immunosuppressive agents (139). In 2000, a co-stimulation blockade-based immunosuppressive therapy was introduced to xenotransplantation by Buhler et al. (140), and has proven more successful than common therapies.

3.1 Glucocorticoids

Glucocorticoids (GCs) belong to the steroid family and were discovered in the 1940s (141). GCs have been used as first-line medication during the induction and maintenance phases after transplantation to prevent acute rejection. In pig-to-primate xenotransplantation, immunosuppressive GC regimens have also been used in most trials (142–144). In an islet xenotransplantation study, all monkeys experienced a normalization of all diabetic and glycemic parameters within four days of GCs administration, suggesting a reversal of diabetes mellitus (145). In another pre-clinical study, the longest survival time (78 days) for a porcine renal xenograft was obtained by applying GCs combined with other immunosuppressants (146).

The 17-hydroxy, 21-carbon steroid configuration characterizing the GC molecules is required for their activity and binding to the GC receptors (GCRs). Changes in this configuration can alter the pharmacodynamic specificities of the GCs (147). GCs' major anti-inflammatory effects result from interferences between activated GCRs and proinflammatory transcription factors such as nuclear factor-κB and activator protein-1 (148). The immunosuppressive mechanisms triggered by GCs include: (i) T cell depletion *via* inhibition of IL-2, which prevents Th1 differentiation and subsequently results in T cell apoptosis; (ii) prevention of B cell clonal expansion through inhibition of the production of IL-2 and related peptides, which reduces antibody production; (iii) induction of eosinophil apoptosis either directly or through IL-5 inhibition (147, 149); (iv) downregulation of the Fc receptors and MHC class II molecules on macrophage surface *via* proinflammatory cytokine inhibition, e.g., IL-1 and TNF-α, and prostaglandins (150); (v) widespread decrease of inflammatory responses in the host by induction of lipocortin-1 (annexin-1) synthesis; (vi) acceleration of lymphocytes apoptosis and abrogation of alloimmune responses to third-party antigens, such as allergens and autoantigens. In conclusion, all immune cells that express GCRs can be modulated by GCs, and as a result, lose abilities such as migration or phagocytosis.

The exact dosage of GCs for induction and maintenance phases varies between different institutions. Methylprednisolone doses vary from 1 to 15 mg/kg, for a duration varying from four days to continuous treatment (143, 146). A metanalysis showed that long-term GCs usage may increase the risk of infections for the hosts (151). Pulse dose steroids are now favoured for treating acute allograft rejection but are less crucial in maintenance immunosuppressive therapy. According to some guidelines for clinical practice, GCs could be discontinued early at first week post transplantation for patients with low immunological risk who receive depleting antibodies as induction therapy (152).

3.2 Calcineurin inhibitors

Two common calcineurin inhibitors (CNIs), cyclosporin and tacrolimus, inhibit the dephosphorylation of nuclear factor of activated T cells (NFAT) by calcineurin, preventing its nuclear translocation and subsequent calcineurin-dependent gene transcription (153, 154). This inhibition results in

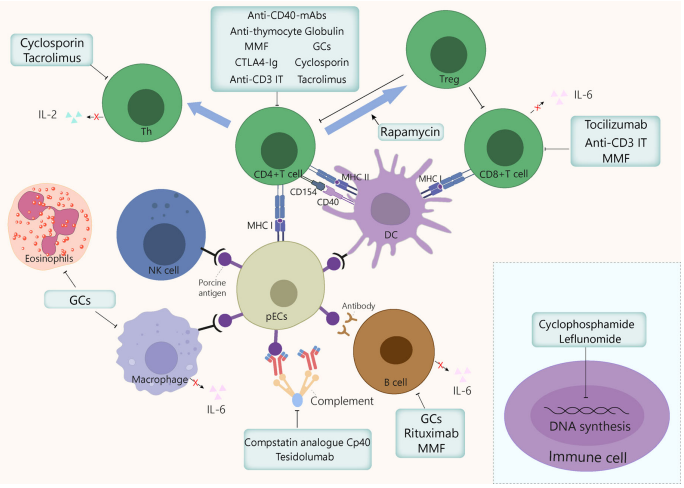

FIGURE 3
Mechanism of action of the immunosuppressants commonly used in xenotransplantation. GCs exert anti-inflammatory and immunosuppressive effects by inhibiting macrophages, eosinophils, T cells, and to a lesser extent, B cells, by binding to GC receptors in cytoplasm. Cyclosporin binds to cyclophilin, then this drug-immunophilin complex binds to calcineurin, which subsequently prevents Th cell activation and IL-2 production, which eventually inhibits T cell clonal proliferation. Tacrolimus inhibits T cells proliferation by binding to FKBP, which inhibits several transcription factors involved in the production of proinflammatory cytokines. Cyclophosphamide blocks DNA alkylation in various cell types, leading to programmed cell death induction and preventing cell division. Leflunomide inhibits the synthesis of pyrimidines, thus arresting cell cycle in S phase. Mycophenolate mofetil prevents T and B cell proliferation by specifically inhibiting a purine pathway required for lymphocyte division. Polyclonal anti-thymocyte globulins are mainly directed against T cells. However, other immune cells sharing common surface antigens with T cells can also be affected to a lesser extent. Monoclonal antibodies target specific cytokine pathways (e.g., IL-6Rα) or cell surface markers, such as CD3, for anti-C3 IT, or CD20, for rituximab. The IL-6 receptor inhibitor tocilizumab reduces systemic inflammation and inhibits of CD8+ T cell and B cell differentiation. Anti-CD3 IT can deplete CD3+ T cells transiently and reduces the number of T cells in circulation and in lymph nodes. Rituximab is a B cell-depleting drug that targets CD20. Rapamycin exerts immunosuppressive and anti-proliferative effects of T cells by inhibiting the activation of S6K1 and PI3 kinase signalling. CTLA-4Ig and anti-CD40mAb target the costimulatory pathways CD80/86:CD28 and CD154:CD40, respectively, thereby dampening T cell activation. The compstatin analogue Cp40 and Tesidolumab inhibits complement C3 and C5 respectively, thereby reducing complement activities. FKBP, FK506 binding protein; GCs, glucocorticoids; IT, immunotoxin; mAb, monoclonal antibody; MMF, mycophenolate mofetil, pECs, porcine endothelial cells.

decreased T cell maturation and lymphokine production, including that of IL-2.

3.2.1 Cyclosporin

Cyclosporin is a cyclic polypeptide consisting of 11 amino acids, most of which are hydrophobic. The discovery of cyclosporin in the early 1980s had a huge impact on the transplantation field by decreasing drastically the rate of acute rejection. This drug binds to cyclophilin; the drug-immunophilin complex then binds to calcineurin, which prevents T cell activation and IL-2 production, thus inhibiting T cell clonal proliferation. Cardiac xenografts treated with steroids and cyclosporin achieved a survival of 77 days, with no signs of hyperacute rejection or cyclosporin-induced malignancies (155). In baboon-to-monkey liver xenotransplantation, two monkeys survived for 91 and 1076 days, respectively, with cyclosporin administered after transplantation, for example, at doses of 3 to 8 mg/kg/day (139), or 20 mg/kg intravenously two hour before transplantation followed by oral administration on next three days (143). In a cardiac xenograft transplantation, the highest cyclosporin blood levels (around 1000 ng/ml) correlated with the highest graft survival rate of the host animals (142).

3.2.2 Tacrolimus

Tacrolimus, a 23-membered macrolide lactone, was isolated for the first in 1987 from *Streptomyces tsukubaensis* (156). It inhibits T cell proliferation by binding FK506 binding protein (FKBP) (157), which inhibits calcineurin by binding it specifically and competitively (158). Subsequently, NFAT nuclear translocation is inhibited, which provokes the downregulation of downstream genes encoding cytokines, including TNF-α, IL-2, IL-3, IL-4, CD40L, IFNγ, and GM-CSF (158, 159). This tacrolimus-induced cascade finally leads to reduced T cell proliferation. Tacrolimus was first approved for liver transplantation in 1994, and since, its used has been extended to become the backbone of immunosuppressive therapy after solid organ transplantation. Later, it has been used for induction and maintenance immunosuppressive therapy, usually in combination with GCs that are then rapidly de-escalated (160). Tacrolimus effectively prevents acute rejection and leads to lower rejection rates and longer rejection-free periods (161, 162). In pig-to-rat islet xenotransplantation model, tacrolimus also exerts a noticeable immunosuppressive effect (163, 164). The oral bioavailability of tacrolimus ranges from 5 to 67% (mean value

of 27%), and its half-life ranges from 3.5 to 40.5 hours (165). Protocols with tacrolimus constantly evolve (166). In a pre-clinical study on pig-to-NHP islet xenotransplantation, tacrolimus was orally administered daily from day −3 to up to day 56 to achieve stable levels (3–6 ng/mL) (164). In another study, tacrolimus was injected intramuscularly twice daily at a dose of 0.05 mg/kg from day −2 and for up to 6 months after transplantation (167). The preferred administration route remains oral rather than sublingual or intramuscular (168). The adverse effects associated with this drug are mainly nephrotoxicity and neurotoxicity, which can be mild to severe. To avoid adverse effects, it is important to maintain tacrolimus at a stable dose. Because the *CYP3A5* genotype is associated with a remarkable impact on tacrolimus pharmacokinetics, it should be considered in the dosing algorithm of this drug (169). Moreover, some research attempted to develop machine-learning models to predict tacrolimus dose stability, which might provide more accurate approaches to achieve personalized medicine in clinics (170).

3.3 Antiproliferative agents

3.3.1 Cyclophosphamide

Cyclophosphamide (CYC) is widely used to prevent transplant rejection and graft-vs-host complications (171). It is a nitrogen mustard drug that affects DNA alkylation in a non-cell cycle phase-specific manner, and is toxic for all human cells to various degrees (172). The active form of CYC inhibits protein synthesis *via* DNA and RNA crosslinking, leading to programmed cell death and prevention of cell division (173).

The immunosuppressive effect of CYC mainly relies on direct deletion of the host's mature T cells that are highly proliferating and reactive to the donor's antigens (174). It also has the capacity to deplete Tregs to counteract immunosuppression in cancer, decrease the production of T cell growth factors, e.g., type I interferons, and precondition host T cells for donor cells, hence attenuating rejection (175). Regimens with CYC in xenotransplantation have proven effective in some cases (146, 176). In pig-to-rhesus corneal transplantation, intravenous injection of CYC followed by pig bone marrow cell transplantation reduced inflammatory cell infiltration (177). CYC is applied typically as a continuous treatment administered orally or intravenously in pulses, with doses ranging from 10 to 40 mg/kg (146, 178). Intermittent intravenous rather than daily oral CYC has been used to minimise bladder and gonadal toxicity. Another side effect of CYC is myelosuppression, which causes leukopenia and neutropenia and can lead to severe and sometimes fatal infections, including viral infections (179).

3.3.2 Mammalian target of rapamycin inhibitors

The mammalian target of rapamycin (mTOR) signalling pathway has important functions in cell growth and metabolism regulation (180). Rapamycin can bind a 12-kDa FK506-binding protein (FKBP12) to form a gain-of-function complex that acts as an allosteric inhibitor of mammalian TOR complex 1 (mTORC1) (181). Rapamycin exerts its immunosuppressive and anti-proliferative properties *via* the inhibition of S6K1, a serine/threonine kinase activated by a variety of agonists (182, 183). In rat-to-mouse islet transplantation, rapamycin could induce Treg-mediated tolerance (184). Moreover, Singh et al. verified that in baboons, treatment with rapamycin increases CD4+ Tregs induction from naïve CD4+ T cells, thereby suppressing anti-porcine xenogeneic response *in vitro* (185). Furthermore, in both allo- and xenotransplantation, graft recipients treated with IL-17-neutralizing antibodies showed the highest percentage of Tregs (186, 187). An example of reported schedule for treatment with rapamycin consists of 0.2 mg/kg during the first three days post-transplantation, followed by treatment every other day until day 14 (184).

3.3.3 Leflunomide

Leflunomide inhibits the dihydro-orotate dehydrogenase, a critical rate-limiting enzyme for pyrimidine synthesis. Therefore, it arrests cell cycle progression from S to G2 phase (188). The literature regarding the role of leflunomide in xenotransplantation is limited but indicates that this drug inhibits rat-to-mouse cardiac xenograft rejection by supressing NF-κB signalling pathway and adaptive immune responses (189).

3.3.4 Mycophenolate mofetil

Mycophenolate mofetil is the semisynthetic morpholinoethyl ester of mycophenolate acid, which prevents T and B cell proliferation by specifically inhibiting a purine pathway required for lymphocyte division (190). MMF, usually administered intravenously at a dose of 20mg/kg twice per day, has been mainly applied together with other immunosuppressants as maintenance regimen and achieved considerable long-term survival of xenograft (the longest reported to be 945 days) in cardiac xenotransplantation (191–194).

3.4 Monoclonal or polyclonal antibodies

Monoclonal antibodies (mAbs) are widely used in clinics and experiments. Most have cell-specific immune-modulatory properties directed for example at CD3+ T cells, which are particularly pathogenic in the context of solid organ transplant rejection (195). Recent studies suggested that in the absence of irradiation or chronic immunosuppressive drugs, renal tolerance can be stably established in primates (18–20) by using an anti-CD3 immunotoxins (ITs) that ablate T cells transiently (145, 196, 197). T cell numbers in blood and lymph nodes could be reduced to 1% of their initial values following anti-CD3 IT depletion, which established long-term tolerance towards mismatched renal allografts (145). Anti-CD3 ITs administered two hours pre-transplantation at a dose of 100 μg/kg and again

on the day following transplantation has demonstrated efficacy (143). However, this schedule should be further verified in the future.

Subsequently, monoclonal antibodies were generated against specific cytokines that play a role in immune cell-mediated toxicity and tissue damage. IL-6 is induced by inflammation and contributes to CD8+ T cell and B cell differentiation (198). In addition, it is a crucial factor in systemic inflammation and endothelial cell survival after xenotransplantation. More recently, Zhao et al. proposed that IL-6 may promote coagulation and inflammation during xenotransplantation (198). Furthermore, Ezzelarab et al. uncovered that biologics inhibiting the IL-6 pathway (e.g., Tocilizumab) could mitigate systemic inflammation in xenograft recipients (SIXR) and may be required to prevent coagulation dysregulation after xenotransplantation (199). Tocilizumab is a biological that blocks human IL-6Rα, and has been considered to reduce inflammation by inactivating the STAT3 pathway acting downstream of IL-6Rα. Tocilizumab was also found to delay the revascularisation of xeno-islets in a pig-to-NHP model (200). Another case report brought exciting results on the use of tocilizumab in combination with other immunosuppressants, which allowed to achieve a 136 day-pig kidney survival (201). However, another recent research by Zhang et al. reported that serum IL-6 increased in baboons receiving tocilizumab before xenotransplantation. This increase could be detrimental to the survival of the pig xenograft by promoting IL-6 binding to pig IL-6R and subsequent pig cell activation (202). Thus, more clinical trials are needed to determine whether tocilizumab is beneficial or detrimental to xenotransplantation. The dose of tocilizumab was consistently 10 mg/kg in all reported cases, with a treatment schedule usually starting on days -1, 7 and 14, followed by administration every two weeks (198).

Another monoclonal antibody used in transplantation is the chimeric anti-CD20mAb rituximab that leads to B cell depletion (203). In addition of being an effective treatment for post-transplant lymphoproliferative disorders, Rituximab can serve as treatment for acute rejection, as some evidence suggested that it could stop the progression towards chronic antibody-mediated rejection (204). Its mechanism of action may be explained by its impact on B cell modulation of the T cell responses, and its long-term effects on plasma cell development (205). In combination with other immunosuppressants, anti-CD20mAb was reported to achieve a 136 day-pig kidney survival (201). An example of reported schedule for treatment with rituximab in transplantation is 19 mg/kg at days -14, -7, 0 and 7 (206).

Polyclonal anti-thymocyte globulins (ATGs) are antibodies obtained by injecting animals, usually rabbits, with human lymphoid cells such as B lymphoblasts, peripheral T cells, or thymocytes, and then harvesting and processing the sera to purified the immunoglobulins (207). ATGs are predominantly directed against T cells, but other immune cells sharing surface antigens with T cells can also be targeted to lesser degrees, as for example B cells, monocytes, and neutrophils. ATG primary mechanism of action consist in promoting lymphocyte depletion through T cell activation-induced apoptosis and complement-dependent lysis (208). In a preclinical study, ATGs were shown to improve engraftment and survival of neonatal porcine xenoislets (209). They can also extend pig kidney survival when combined with other immunosuppressants (201). ATGs are usually prescribed before transplantation at a dose of 10 mg/kg on day –3 (201), or at a dose of 5 mg/kg on days –2 and –1 (206).

3.5 Blockade of costimulatory signals

3.5.1 Blockade of CD80/86:CD28 costimulatory pathway by CTLA4Ig

T cell activation requires co-stimulation *via* engagement of CD28 on the T cell with CD80/86 on the APC. Cytotoxic T lymphocyte–associated protein 4 (CTLA4) is a competitive inhibitor of CD80/86 that downregulates T cell responses (210). This T cell suppressive activity served to engineer a human IgG heavy chains coupled with CTLA4 to create a fusion antibody able to prevent graft rejection (211). The new generation CTLA4Ig, belatacept, displayed a significantly higher affinity for CD80/86 in a pre-clinical renal transplantation model in primate (212), and showed greater efficacy in modulating adaptive immune responses (213, 214). Belatacept proved able to decrease the antigraft humoral immune response in intracerebral transplantation of mesencephalic pig xenografts into primates (215). *In vivo*, CTLA4Ig is able to dampen T cell-dependent immune responses and prolong long-term xeno- and allograft survival (216–218). Levisetti et al. reported that two out of five CTLA4Ig-treated monkeys showed prolonged graft survival, while the humoral responses were suppressed in all treated animals (219). Buerck et al. and his group generated a novel transgenic (tg) pig line expressing the CTLA-4Ig analogue LEA29Y and demonstrated that transplanted INSLEA29Y-tg porcine neonatal porcine islet-like clusters (NPICCs) displayed normal beta cell function and survived from rapid T lymphocyte-mediated rejection during 30-day observation period. However, the long-term effect regarding xenograft rejection still remained unknown (220). In another pig to baboon clinical trial, the blockade of CD28-B7 costimulation pathway using human CTLA4Ig has been shown unsuccessful to prevent xenograft rejection, making the role of CTLA4Ig controversial (221).

3.5.2 Targeting of CD154:CD40 costimulatory signal with anti-CD40mAb

The interaction between CD154 on activated T cells and CD40 on APCs results in CD80/86 upregulation on APCs, enhancing another component of T cell co-stimulation (222). CD154 is also found on platelets, and not surprisingly drugs targeting CD154 are

associated with an increased risk of thrombosis in primate (223). Thus, the focus of drug development has been changed to target CD40 and several anti-CD40mAbs are under development. Blockade of CD40/CD154 signaling by anti-CD40mAb was shown to prolong graft survival and suppress xenograft rejection (192, 224). Among these, a fully humanized anti-CD40mAb, iscalimab, appears to be a promising candidate in transplantation (225). Pre-clinical application using immunosuppressive anti-CD40mAb 2C10R4 combined with tacrolimus in pig-to-NHP islet xenotransplantation was effective in prolonging islet graft survival (164). In pig-to-mouse islet xenotransplantation, short-term administration of the anti-CD40mAb MR-1 and the anti-LFA-1mAb increased the survival of neonatal porcine islets (226). Interestingly, the short-term use of MR-1 alone prolonged porcine islet graft survival and promoted CD4+ Tregs recruitment into the graft and secondary lymphoid tissues (227). Consistently, lower numbers of CD4+ Tregs increased the risk of rejection in cardiac xenotransplantation in a pig-to-NHP model (228). For treatment, anti-CD40mAb was reported to be infused intravenously at a dose of 20–50 mg/kg on days −4, 0, 4, 7, 10 and 14 of transplantation, followed by weekly infusion for three months, and biweekly fusion thereafter (164). Alternatively, anti-CD40mAb could be used at a dose of 50 mg/kg on days −1, 0, 5, 9, and 14 (206).

3.6 Complement inhibition

As discussed above, complement activation is involved at every stage of xenograft rejection. Thus, a complementary approach is to administer agents that either deplete or inhibit complement activation. Many interventions have been introduced to prevent complement-mediated injuries during xenotransplantation (229, 230). Among these, cobra venom factor extends graft survival significantly in allotransplantation, albeit it only has a temporary effect (231). C1-esterase inhibitor has been reported to be active in NHPs and was recommended to replace cobra venom factor as complement inhibitor (232, 233). The compstatin analogue Cp40, a newly developed potent inhibitor of complement C3, inhibits leukocytes adhesion and neutrophils attachment to porcine endothelium (230). Moreover, Cp40 inhibits pECs and leukocytes activation. It reduces the levels of adhesion molecules such as E-selectin, ICAM-1, ICAM-2, and VCAM-1 on pECs, and of the integrin CD11b on neutrophils, paving the way for future therapeutic interventions targeting complement activities (230). Schmitz et al. reported that Cp40 could significantly prolong median allograft survival time in an NHP model. Normal kidney function was maintained at 50% in Cp40-treated primates after the last day of treatment (234). In another case report, Tibetan macaques receiving liver xenografts with immunosuppressors, including Cp40, did not exhibit severe coagulation disorders or immune rejection (235). Another complement inhibitor, the anti-C5 antibody Tesidolumab, has been recently reported to reduce early antibody-mediated rejection and prolong survival in renal xenotransplantation (236). Cp40 was used at a dose of 2 mg/kg three times daily, on day 2 prior to kidney transplantation and day 14 after kidney transplantation (234). Yet, the most appropriate dosages need to be determined more precisely. Tesidolumab was given at a dose of 30 mg/kg on the day of transplant, followed by weekly intravenous injection at 10 mg/kg for seven weeks, at which point anti-C5 was discontinued.

3.7 Genetic engineering strategies

Currently we are able to create new genetic modifications of the porcine genome (over 40 genetic variants to date), hoping to achieve better survival of the xenografts. The multiplex creation of a *GGTA1/CMAH/B4GalNT2* KO pig has shown the ability to reduce antibody mediated rejection in humans. There are also other extensive genome engineered pigs which have greater compatibility with the human immune system. For example, the generation of porcine endogenous retroviruses (PERVs) KO·3KO·9TG (hCD46, hCD55, hCD59, hTHBD, hTFPI, hCD39, hB2M, HLA-E and hCD47) pig enhances the pigs' immunological compatibility and blood-coagulation compatibility with humans (64, 237–240). Recombinant expression of human complement regulatory molecules hCD59 and hDAF on porcine articular chondrocytes could also prevent humoral rejection in cartilage repair (241). By using CRISPR/Cas9 system, Sake et al. tried to abrogate MHC-I expression on xenografts to silence T-cell and NK cell-mediated cell lysis (64). Four genetic pigs died within the first days due to weakness, and the remaining two piglets developed acute fevers at an age of 3-4 weeks leading to sudden death (64). Xie et al. succeeded in alleviating antibody-mediated rejection using Gabarapl1 knockdowns in primary porcine aortic endothelial cells (PAECs) (238). While in another pre-clinical trial, the inhibition of COX-2 expression decreased PAECs death from 20% to 7% after 2 hours, making COX-2 inhibitors a candidate for therapeutic targeting to protect vascular endothelial cells in xenotransplantation (43). More is not always better, extensive genetic engineering can lead to congenital malformations/decrease animal viability (242, 243). What exact genetic modifications do we need in the organ-source pig should be fully considered in the future.

It is noteworthy that researchers are investigating alternative options. One of them is to grow complex tissues or organs using the body's own regenerative capacity. Masano et al. tried to use a xenogeneic animal as an *in vivo* bioreactor to promote regeneration of a liver graft and successfully acquired fully regenerated small liver grafts under appropriate immunosuppressive therapy (244). This alternative option has fewer ethical concerns, but before it can be considered further, more research are needed to reduce complications and tested in larger animal models.

4 Organ-specific barriers and challenges

In recent years, a number of pig-to-NHP preclinical xenotransplantation studies have been performed with various organs. While HAR has been alleviated owing to gene-editing technologies, DXR and chronic rejection remain urgent issues to be solved. Another concern focuses on PERVs transmission, which is a major hurdle to the clinical use of pig cells, tissues, and organs for treatment of organ failure in humans. However, it is still uncertain whether PERVs is pathogenic to humans, or if it could recombine with hERVs to form new viruses. Genetic engineering techniques such as CRIPSR/Cas9 genome editing system could prevent their activation or delete them from the pig cells (245, 246). Further, the ethical issues around xenotransplantation have not been sufficiently discussed. Strict medical and ethical guidelines and regulations are needed before clinical applications can be tried on selected patients. Beyond the common ethical and technical issues shared by the different areas of xenotransplantation, there are also organ-specific barriers that are briefly addressed below.

4.1 Islet xenotransplantation

Islet xenotransplantation is a promising alternative approach to Type 1 Diabetes (T1D) treatment and has achieved long-term normoglycemia in porcine-to-primate studies (247–249). Based on preliminary studies in NHPs, the first case of clinical islet xenotransplantation to human can be traced back to 1994 (250). Currently, clinical trials using islet xenotransplantation are developing more rapidly than those in other xenotransplantation areas. In 2014, a clinical trial using islet xenotransplantation under regulatory framework was registered at ClinicalTrial.gov (251). This was followed by Phase I/IIa and IIb clinical trials using encapsulated neonatal porcine islets in xenotransplantation performed in Argentina (252), which resulted in a mean transplant estimated function of approximately 0.5, with transplants maintained for more than two years, and a significant reduction in the number of unaware hypoglycemia episodes. Surely, there are still many problems to be solved for islet xenotransplantation before reaching their clinical use, including physiological function consistency, immune rejection, islet loss, and prevention of PERVs infection (253). Immune rejections included HAR, mediated by Gal and non-Gal antigens (254), instant blood-mediated inflammatory reaction (IBMIR) that may have provoked 60–80% of islet loss (255), and $CD4^+$ T cell-mediated cellular rejection that plays a major role in islet destruction (256). Strategies to alleviate rejection mainly include islet encapsulation and gene editing technology. Recent clinical trials mainly focused on encapsulating the neonatal porcine islets in different high molecular compounds (257). While cell encapsulation technology can potentially shield the islets from the host's immune rejection at initial stage, long-term therapeutic efficacy is still a challenge (258). Another hot spot of research is to attempt different transplantation sites, including the portal vein, subrenal capsule, subcutaneously, the muscle, spleen, gastric submucosal space and peritoneum, depending on the islet volume and its naked or encapsulated status, in order to reduce islet loss (259, 260). There is still no optimal site for transplantation, but the peritoneal cavity is favoured in clinical trials.

4.2 Liver xenotransplantation

Liver xenotransplantation from chimpanzee to human was first held in the 1960s (261). More trials were carried out in the 1990s (262), which remained ultimately unsuccessful. The transplanted patients either died from sepsis due excessive immunosuppression, or from hepatic failure with clear rejection. These failures terminated the attempts of liver xenotransplantation in clinical application. Until today, the research has mainly focused on pig liver xenotransplantation to NHPs. Shah et al. (263) have recorded, so far, the longest survival time after xenotransplantation of a pig livers to NHPs, which was of 25 days, using with an *α1,3-galactosyltransferase* knockout miniature swine as a donor. Two major problems must be overcome in liver xenotransplantation: lethal thrombocytopenia and antibody-mediated rejection (AMR) targeting antigens such as *α1,3GT*, N- glycolylneuraminic acid and *β4GALNT2* (235). Moreover, hepatic cold-induced injuries are also a serious concern (264). AMR has now been reduced by using gene editing technology including CRISPR/Cas9, TALEN, and other genome editing and transgenic methods (254). To solve hepatic cold-induced injuries, Li et al. succeeded to increase porcine hepatocyte viability by optimizing spheroid cold storage conditions under four different cold storage solutions (265). Despite these efforts, the graft survival is limited by either the development of a thrombotic microangiopathy and/or consumptive coagulopathy (266, 267). Cross-species thromboregulation becomes more complicated in case of liver xenotransplantation because the liver produces most coagulation factors. Current preclinical studies are dedicated to elucidating the immunobiology behind platelet activation, aggregation, and phagocytosis, especially during interactions between platelets and liver sinusoidal endothelial cells, hepatocytes, and Kupffer cells (28, 268). We believe that if the severe and immediate thrombocytopenia could be prevented, pig liver xenotransplantation could be used as a bridge towards allotransplantation.

4.3 Cardiac xenotransplantation

The first attempt of pig-to-NHP cardiac xenotransplantation (CXTx) started in the mid-1980s (269). Currently, it is the standard model to conduct preclinical xenotransplantations. A major breakthrough came with the introduction of the genetic deletion of the *α1,3GT* gene in 2003, which reduces HAR to a large extent.

The longest survival of heterotopic heart xenograft, reaching up to 945 days, has been achieve in baboons, using cardiac xenografts from *GTKO.hCD46.hTBM* pigs, with ATG and anti-CD20 antibody treatments, followed by maintenance with MMF and high-dose anti-CD40 immunosuppressive regimen (193). The first clinical trial of pig to human CXTx was carried out with genetically modified pig heart transplanted into a 57-year-old man in the USA in 2022, and the patient survived for two months without signs of rejection, while the cause of death is unknown (270). Despite many breakthroughs on different aspects of xenotransplantation in recent years, there are still barriers to be overcome before large scale clinical CXTx can be conducted, including immunological barriers, perioperative cardiac xenograft dysfunction (PCXD), detrimental xenograft growth, and PERV infection. PCXD is unique to orthotopic CXTx and has not been observed in heterotopic CXTx. It can cause xenograft failure within the first 48 hours (194). The exact mechanism of PCXD is unclear but may stem in incompatibilities between porcine and primate plasma, the latter carrying non-Gal antibodies (271). Cold non-ischemic continuous perfusion of the donor's heart with STEEN solution (a buffered extracellular solution) appears to be an effective way to alleviate PCXD (233, 272). The detrimental xenograft overgrowth occurring after CXTx leads to diastolic dysfunction and congestive liver damage. The overgrowth could be inhibited by lowering baboons' blood pressure to match that in pigs' heart, by reducing the use of cortisone early, or by using temsirolimus, as in a particular study (233). This latter strategy has not tested by other researchers. The relevant ethical issues around CXTx have not been completely defined yet. Strict medical and ethical guidelines and regulations are still needed before proceeding towards clinical application to selected patients.

4.4 Kidney xenotransplantation

Kidney xenotransplantation has a long and largely unsuccessful history. The first clinical trial was carried out in 1905, when slices of rabbit kidneys were inserted into a child, who died 16 days later due to pulmonary congestion. Reemtsma et al. transplanted pairs of chimpanzee kidneys into six patients in 1964, with the longest survival reaching nine months (273). Later attempts using monkeys and baboons as source of kidneys were even less successful. In majority, the deaths occurring in these clinical studies were related to either rejections or infections. These disappointing results terminated the clinical application of kidney xenotransplantation. However, researchers have re-explored this possibility later, due to shortage of available kidneys, and pig-to-NHP xenotransplantation has now become a standard experimental model. Similar to CXTx, rapid progress has been made since 2005, with the availability of genetically engineered pigs (274). In 2015, two groups reported the survival of life-supporting genetically engineered pig kidneys for > 4 months, maintained by a treatment involving new immunosuppressive agents blocking T cell co-stimulation (201). Based on this progress, a new clinical trial with two GTKO porcine kidneys transplanted to a brain-dead patient on a ventilator has been carried out in 2021. The kidneys were connected outside of the body to blood vessels on the patient's legs and monitored over a period of 72 hours (275). No HAR and no transmission of porcine retroviruses were detected, and the kidneys produced variable amounts of urine, but creatinine clearance was not recovered. Although this attempt surely brought substantial information and improvement for kidney xenotransplantation, there is still a long way to make kidney xenotransplantation possible in humans. In this case, the host was brain dead and artificially maintained, and therefore cannot been considered a living body. This trial is close to a clinical trial, but it still cannot be considered as such. We would consider this attempt as a bridge between xenotransplantation trials in animals and clinical trials in humans. Organ-specific problems linked to kidney xenotransplantation include hypovolemia syndrome, erythropoietin function-associated anaemia, and rapid growth of the pig kidneys after transplantation (199). The primate organisms are not aware of the fluid loss occurring during hypovolemia syndrome, which may result from a dysfunction of the renin-angiotensinogen system. This could be avoided by conserving the native kidneys *in situ* (276). It is difficult to assess whether the pig erythropoietin functions adequately in primates, but pigs genetically engineered to produce human erythropoietin may solve this issue (277). Kidneys transplanted from a strain of pig grow early and rapidly in primates. This phenomenon may result from an innate factor, and could also be solved by knocking out the gene encoding the growth hormone receptors of the pigs (278). Finally, the inclusion criteria for the selection of patient candidate to kidney xenotransplantation is more difficult than in other areas, as dialysis represents a therapeutic option for these patients. Therefore, until proven safer and more efficient, kidney xenotransplantation cannot be considered.

5 Conclusion and perspectives

The field of xenotransplantation has been progressing rapidly with many breakthrough achievements in recent years. However, there are still several problems ahead of its use in clinical practice: (i) although porcine-to-human xenotransplantation of kidneys and hearts have been carried out, it is not known when xenotransplantation of liver, small intestine, and even pancreas will become possible. (ii) NHPs are phylogenetically close to humans and share many physiological, anatomical, immunological, and neurological similarities, making them excellent experimental models for research (279). However, there are still differences between species, and it is not clear to which extent studies in NHPs can fully predict xenorejection and the clinical outcome in humans. (iii) Until now, the survival of transplanted organs on the long term largely depends on high doses of different immunosuppressants, which would expose the recipients to high infection risks and other side effects. Although

more and more genetically engineered pigs are created by gene-editing technologies, the question remains as to whether it will become possible to achieve long-time survival without immunosuppressants, by reducing the immunogenicity of the transgenic donors. Ethical aspects of xenotransplantation have been discussed for many years (280), with particular considerations on issues related to the risks for patient with xenograft do develop and propagate porcine infections (281). (iv) While CRISPR/Cas9 genome editing can remove the PERV gene from pig, the risk of xenozoonosis with other roseoloviruses remains (282). For example, porcine cytomegalovirus was the cause of a significant reduction of the survival time of the transplanted pig organs (283). However, such concerns can also be appropriately handled with modern drug therapies, selective breeding, and genetic modification.

As allotransplantation is restricted due to cell and organ shortage, xenograft provides an alternative source of tissues, and xenotransplantation may represent the next revolution in medicine. More patients with end-organ failure would undoubtedly benefit from breaking the immunological barriers to xenotransplantation in near future.

Author contributions

YW, CC and SD conceived the idea. QZho and TL wrote the manuscript. KW and QZha prepared the figures. CC, KW, ZG, CC and YW revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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