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Donor-Derived Cell-Free DNA (dd-cfDNA) as an Early Noninvasive Biomarker of Graft Injury in Pig-to-Monkey Islet Xenotransplantation

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ABSTRACT

Background: In pig-to-nonhuman primate islet transplantation, reliable, sensitive biomarkers are needed to detect graft damage at an early stage before irreversible islet loss occurs. In our study, we investigated donor-derived cell-free DNA (dd-cfDNA) as an early, noninvasive biomarker of graft injury by analyzing its correlation with porcine C-peptide levels, complement activation markers, and donor-specific antibodies (DSAs).

Methods: Streptozotocin-induced diabetic cynomolgus monkeys received 50 000–100 000 IEQ/kg of intraportal islets from quadruple-knockout (QKO; *GGTA1*, *CMAH*, *B4GALNT2*, and *A3GALT2*) pigs. Cohort 1 received antithymocyte globulin (ATG), tacrolimus, mycophenolate mofetil (MMF), and anti-inflammatory agents (i.e., anakinra, adalimumab, and tocilizumab), whereas Cohort 2 received the same regimen plus rituximab and crovalimab. Graft function and immune responses were assessed by measuring porcine C-peptide levels, complement activation markers, histology, and dd-cfDNA kinetics.

Results: Cohort 1 showed transient porcine C-peptide secretion with marked dd-cfDNA elevation at 7 d postoperatively that coincided with complement activation (i.e., C5a and membrane attack complex (MAC)) and dense CD3^{+} T-cell and CD68^{+} macrophage infiltration, which resulted in early graft loss. Cohort 2 maintained stable C-peptide levels, lower dd-cfDNA levels, and reduced complement activation with improved graft preservation. Moreover, dd-cfDNA correlated negatively with C-peptide and positively with C5a but not with MAC. In both cohorts, DSA levels remained unchanged.

Conclusions: Our study revealed that dd-cfDNA levels correlate with graft damage and C5a in QKO porcine islet xenografts, which corroborates dd-cfDNA utility as an early biomarker for predicting instant blood-mediated inflammatory reaction (IBMIR). These findings indicate that dd-cfDNA may be able to detect early islet xenograft damage.

Ji-Jing Yan & Jong-Min Kim contributed equally to this work

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1 | Introduction

Porcine xenotransplantation offers a promising solution to the critical shortage of human donor organs and tissues. Although pancreatic islet transplantation can restore insulin production in patients with type 1 diabetes mellitus, limited donor availability remains a major barrier to its clinical application [1, 2]. Porcine islets provide an alternative and scalable source, one with physiological compatibility and that may tolerate genetic modification [3, 4].

Preclinical studies in nonhuman primates (NHPs) have demonstrated successful engraftment and metabolic control in porcine islet xenotransplantation, which supports its potential as a viable therapeutic strategy [5]. However, early graft loss caused by innate immune activation, particularly the instant blood-mediated inflammatory reaction (IBMIR), remains a major obstacle to long-term graft survival. IBMIR, characterized by the rapid activation of coagulation and complement cascades accompanied by inflammatory cell infiltration, leads to substantial early islet loss during the peritransplant period [6–9].

To overcome those innate immune barriers, the genetic modification of donor pigs has been instrumental. Natural antibodies against porcine carbohydrate antigens such as α-Gal, Neu5Gc, and SDa contribute to early graft injury by amplifying IBMIR and inducing hyperacute rejection as in xenotransplantation. The knockout of those antigenic targets (i.e., *GGTA1*, *CMAH*, *β4GALNT2*, and *A3GALT2*) significantly reduces antibody binding and mitigates early inflammatory responses [10–13]. Furthermore, the expression of human complement regulatory proteins (e.g., hCD46, hCD55, and hCD59) on porcine islets provides additional protection by attenuating complement activation and IBMIR, which reduces early islet loss and improve graft engraftment [14–16].

Despite those advances, early graft injury remains a challenge and underscores the need for reliable, noninvasive biomarkers for the early detection of xenograft damage. Donor-derived cell-free DNA (dd-cfDNA), representing DNA fragments released into the recipient's circulation from injured donor graft cells, has recently emerged as a promising biomarker in various fields, including oncology [17, 18] and solid organ transplantation for the early detection of tissue injury and rejection [19–21]. In islet xenotransplantation, studies have demonstrated the use of dd-cfDNA measurement as a biomarker for the death of transplanted islet cells [22, 23]. However, the associations between dd-cfDNA and xenograft function, complement activity, donor-specific antibodies (DSAs), and the severity of rejection have yet to be adequately assessed.

In our study, we used genetically engineered quadruple knockout (QKO; *GGTA1*, *CMAH*, *β4GALNT2*, and *A3GALT2*) pigs as islet donors in a pig-to-NHP xenotransplantation model. With that model, we investigated the potential of using dd-cfDNA as an early, noninvasive biomarker of graft injury by analyzing its correlation with porcine C-peptide levels, complement activation markers, coagulation factors, inflammatory activity, and DSAs. Our ultimate goal was to confirm or disconfirm dd-cfDNA as a predictive marker for early graft injury and a valuable tool for monitoring long-term graft survival in islet xenotransplantation.

2 | Materials and Methods

2.1 | Animals

Pigs genetically modified to four knockout genotypes—α-1,3-galactosyltransferase (*GGTA1*), cytidine monophosphate-N-acetylneuraminic acid hydroxylase (*CMAH*), β1,4-N-acetylgalactosaminyltransferase (*B4GALNT2*), and alpha-1,3-galactosyltransferase2 (*A3GALT2*), collectively called "QKO pigs"—were supplied by Optipharm Inc. (Cheongju, South Korea) and served as the source of donor islets. Five male cynomolgus monkeys 4–5 years old and weighing 4.2–5.6 kg were used as islet transplantation recipients (IDs: 23CM10, 23CM11, 23CM07, 23CM09, and 23CM20). The experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Biomedical Research Institute at Seoul National University Hospital (IACUC No. 23-0025-S1A3).

2.2 | Pig Islet Isolation and Islet Quality Assurance

After genetically modified pigs underwent total pancreatectomy in an operating room under sterile conditions, islets were isolated using the modified Ricordi islet isolation method [24]. Pancreases were distended through the pancreatic duct using Hank's balanced salt solution containing the enzyme Collagenase AF-1 GMP Grade with Neutral Protease AF GMP Grade (Nordmark Biochemicals, Uestersen, Germany). Islet quality was measured using dithizone staining, glucose-stimulated insulin secretion, and acridine orange–propidium iodide staining. For transplantation, we used islets with greater than 80% purity, greater than 90% viability, and a stimulation index exceeding 3.0.

Pig islets were isolated from two QKO pigs at a time, pooled, and infused into the liver of diabetic recipient monkeys through a jejunal vein. Monkeys 23CM10 and 23CM11 received 50 000–60 000 IEQ/kg, whereas monkeys 23CM07, 23CM09, and 23CM20 received 100 000 IEQ/kg.

2.3 | Induction of Diabetes and Islet Transplantation Into NHPs

A central venous catheter was inserted into the right internal jugular vein of each monkey, and a gastrostomy tube was implanted for oral drug delivery, as previously described [25, 26]. Diabetes was induced with streptozotocin (STZ, 110 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) administered intravenously over 5 min following overnight fasting and prehydration. Butorphanol or metoclopramide was also administered to prevent STZ-induced vomiting. Diabetes induction was confirmed by persistent hyperglycemia (i.e., > 300 mg/dL), fasting C-peptide less than 1 ng/mL, and the absence of a C-peptide response in IVGTT and AST testing [26]. Blood glucose was monitored 2 or 3 times daily, and insulin therapy using Novolin N (Green Cross, Yongin, South Korea) and Lantus (Sanofi-Aventis, Paris, France)

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was administered to maintain fasting and postprandial glucose levels less than 150 and 200 mg/dL, respectively.

For transplantation, animals were fasted for 12 h. A midline laparotomy was performed, and islets suspended in heparinized solution (100 IU/kg) were infused into a jejunal vein over 8–12 min, thereby allowing distribution to the liver via the portal system. The vessel was ligated after infusion. Postoperatively, ketamine (50 µg/kg/min), and lidocaine (0.6 mg/kg/h) were infused for 3d, with meloxicam and butorphanol for analgesia. A tether system was used to maintain intravenous fluids and low-dose dextrose infusion as needed. Exogenous insulin therapy was maintained throughout the posttransplant period to achieve target glycemic control, and insulin independence was not defined as an endpoint of this study. Accordingly, because exogenous insulin administration was intentionally continued, graft function was primarily assessed by porcine C-peptide secretion rather than by the achievement of normoglycemia or insulin independence.

Porcine C-peptide in nonhuman primate serum were quantified using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Mercodia AB, Uppsala, Sweden) according to the manufacturer's instructions.

2.4 | Immunosuppressive and Supportive Therapy

Recipient cynomolgus monkeys received a combined immunosuppressive regimen without costimulatory blockade (i.e., anti-CD154). Thymoglobulin, an anti-thymocyte globulin (ATG, 5 mg/kg; Genzyme, Cambridge, MA), was administered during induction to achieve T-cell depletion before islet infusion. Maintenance therapy consisted of tacrolimus (Advagraf; Astellas Pharma Korea, Seoul, South Korea) and either mycophenolate mofetil (MMF, Cellcept; Korea Roche, Seoul, South Korea) or sirolimus (Rapamune; Astellas Pharma Korea). Rituximab was administered for B-cell depletion to further reduce humoral immune responses. To limit instantaneous IBMIR and complement activation, anakinra (Kineret; Amgen, Seoul, South Korea), adalimumab (Humira; Abbott Laboratories Ltd., Queenborough, UK), tocilizumab (Actemra; JW Pharmaceutical Corp., Seoul, South Korea), and two complement inhibitors—the complement-depleting agent CVF (ID: 23CM09) and the C5 inhibitor crovalimab (ID: 23CM20; provided Dr. Kim, Asan Medical Center, Seoul, South Korea)—were administered during transplantation. Heparin was administered during islet infusion to prevent coagulation and thrombus formation.

The monkeys were assigned to two cohorts. Cohort 1 received ATG, tacrolimus, MMF, and anti-inflammatory agents (i.e., anakinra, adalimumab, and tocilizumab), whereas Cohort 2 received the same regimen with additional rituximab and the two complement inhibitors (Figure 1).

2.5 | ELISA

To measure serum C-peptide concentrations, blood samples were first collected in serum-separating tubes. To monitor islet graft loss, porcine C-peptide levels in recipient monkey serum were quantified using a commercial ELISA kit (Mercodia AB, Uppsala, Sweden) according to the manufacturer's instructions.

Monkey peripheral blood samples were collected in heparin tubes and centrifuged at 2000 rpm for 20 min, and the plasma was stored at −80°C until analysis. Cytokines (i.e., TNF-α and IL-6), coagulation factors (i.e., tissue factor [TF] and the thrombin–antithrombin complex [TAT]), and complements (i.e., C3a, C5a, and membrane attack complex [MAC]) were measured in plasma using commercially available ELISA kits (MyBioSource, San Diego, CA, USA) according to the manufacturer's instructions.

2.6 | Histopathological Examination

Liver biopsy samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 4 µm thickness for histological analysis. All slides were immunostained using a DAB chromogen (i.e., brown) and alkaline phosphatase (AP) chromogen (i.e., red). Sections were deparaffinized, followed by antigen retrieval, and the sections were incubated with 3% hydrogen peroxide to block endogenous peroxidase activity as well as blocked with 10% goat serum. First step, slides were incubated overnight with the primary antibody against insulin, followed by detection using the Polink-2 Plus AP Mouse Permanent Red Detection Kit (OriGene Technologies, Rockville, USA). Second step, slides were blocked with 3% hydrogen peroxide and 10% goat serum and subsequently incubated for 2 h at room temperature with the primary antibody (i.e., CD3 or CD68). Detection was performed using the Polink-2 Plus HRP DAB Detection Kit (OriGene Technologies), and slides were counterstained with hematoxylin. Images were captured with a ZEISS Axioscan7 Microscope Slide Scanner (Carl Zeiss Microscopy Deutschland GmbH, Oberkochen, Germany). Cell counting and the analysis of positive cells and area were performed using Image J software, which counted four high-power fields (200 ×) per section. All histologic analyses were conducted by two independent researchers who were blinded to the treatment groups.

2.7 | Detection of Circulating dd-cfDNA

Recipient monkey plasma samples were collected in Cell-Free DNA BCT tubes (Streck,

La Vista, NJ, USA) after pig islet transplantation and shipped to Macrogen, Inc. (Seoul, South Korea) for xeno dd-cfDNA analysis. Library preparation, sequencing, and bioinformatics were performed by Macrogen using the xGen cfDNA Library Prep Kit (Integrated DNA Technologies, Inc. Coralville, IA, USA) and the Illumina NovaSeq X platform. cfDNA libraries were generated from 10 ng of input DNA, adapter-ligated, PCR-amplified with dual indices, and sequenced. Paired-end reads were trimmed and aligned to the Sscrofa11.1 and Macaca_fascicularis_6.0 reference genomes using Bowtie2 and mapped reads were quantified with SAMtools flagstat to calculate the proportion of dd-cfDNA. All analyses were performed in a blinded manner without access to biological or histological data.

To establish baseline dd-cfDNA levels, recipient plasma samples collected 21 days prior to islet transplantation were analyzed. The

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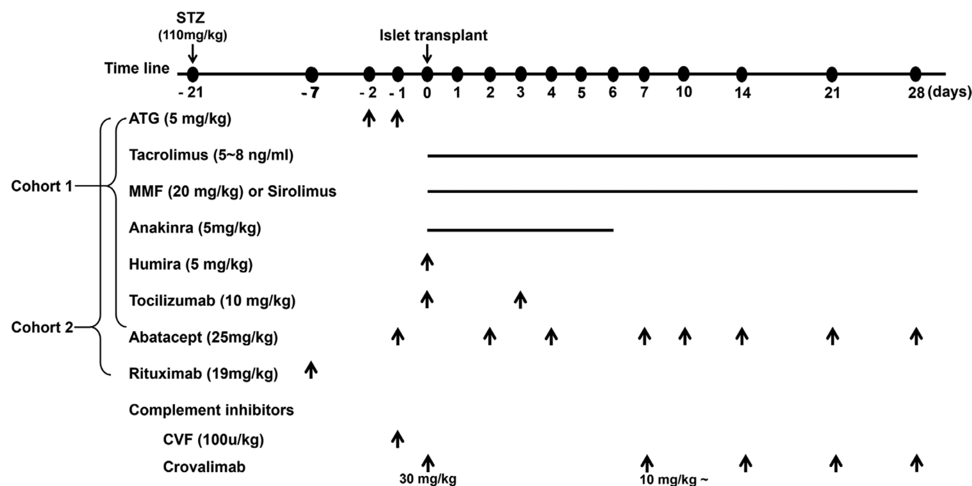

FIGURE 1 | Immunosuppression protocol timelines and treatment schedules. Timeline of immunosuppressive regimens used in QKO porcine islet xenotransplantation. Baseline therapy included ATG, adalimumab, tocilizumab, anakinra, tacrolimus, and MMF or sirolimus, with heparin administered pretransplantation. Cohort 2 additionally received rituximab and crovalimab. ATG, anti-thymocyte globulin; MMF, mycophenolate mofetil; QKO, quadruple knockout.

TABLE 1 | Recipient and islet characteristics.

Donor	Cohort	Recipient	Weight (kg)	IEQ/kg ($\times 10^4$)	Treatment	
QKO pig (GGTA1, CMAH, B4GALNT2, A3GALT2)	1	23CM11	4.9	5	ATG	Tacrolimus MMF anakinra humira tocilizumab
		23CM10	5.0	6		
		23CM07	4.4	10		
	2	23CM09	5.6	10	ATG rituximab crovalimab	
		23CM20	4.2	10		

Abbreviations: ATG, Anti-thymocyte globulin; IEQ, Islet equivalent; MMF, Mycophenolate mofetil; QKO, quadruple knockout.

low-level dd-cfDNA signal detected before transplantation was considered background and used as the baseline reference for all posttransplant analyses.

2.8 | Statistical Analysis

Data are presented as the mean with the standard error of the mean ($M \pm SEM$). Between-group comparisons were performed using Student's t - test, and correlations between variables were assessed using Pearson's correlation coefficient. A p-value less than 0.05 was considered to indicate statistical significance. All analyses were performed using GraphPad Prism (version 10.0; GraphPad Software, La Jolla, CA, USA).

3 | Results

3.1 | Enhanced Early Islet Graft Function and Prolonged C-Peptide Secretion in Cohort 2

Baseline recipient and islet characteristics are summarized in Table 1. Four hours after transplantation, porcine C-peptide levels in the plasma of Cohort 1 recipients rose sharply to above 4 ng/mL. In 23CM10 and 23CM11, C-peptide levels declined rapidly and became nearly undetectable 2 weeks posttransplantation; by contrast, levels decreased to 0.1 ng/mL at 4 weeks in 23CM07 (Figure 2A). At the same time, Cohort 2 showed a lower peak level of approximately 0.6 ng/mL, but C-peptide levels were maintained at 0.2–0.4 ng/mL for up to 4 weeks after transplantation (Figure 2B).

High levels of porcine C-peptide in plasma during the early posttransplant period reflect the rapid loss of islet mass, likely due to IBMIR-mediated injury. In Cohort 2, the administration of rituximab and complement inhibitors appears to have attenuated early islet destruction, which promoted more favorable initial engraftment. Adding abatacept would also be expected to contribute to improved graft maintenance during the later phase of transplantation.

3.2 | Complement Responses and Coagulation Markers Following Porcine Islet Transplantation

An analysis of coagulation markers and complement factors was performed to assess mechanisms of systemic rejection (Figure 3).

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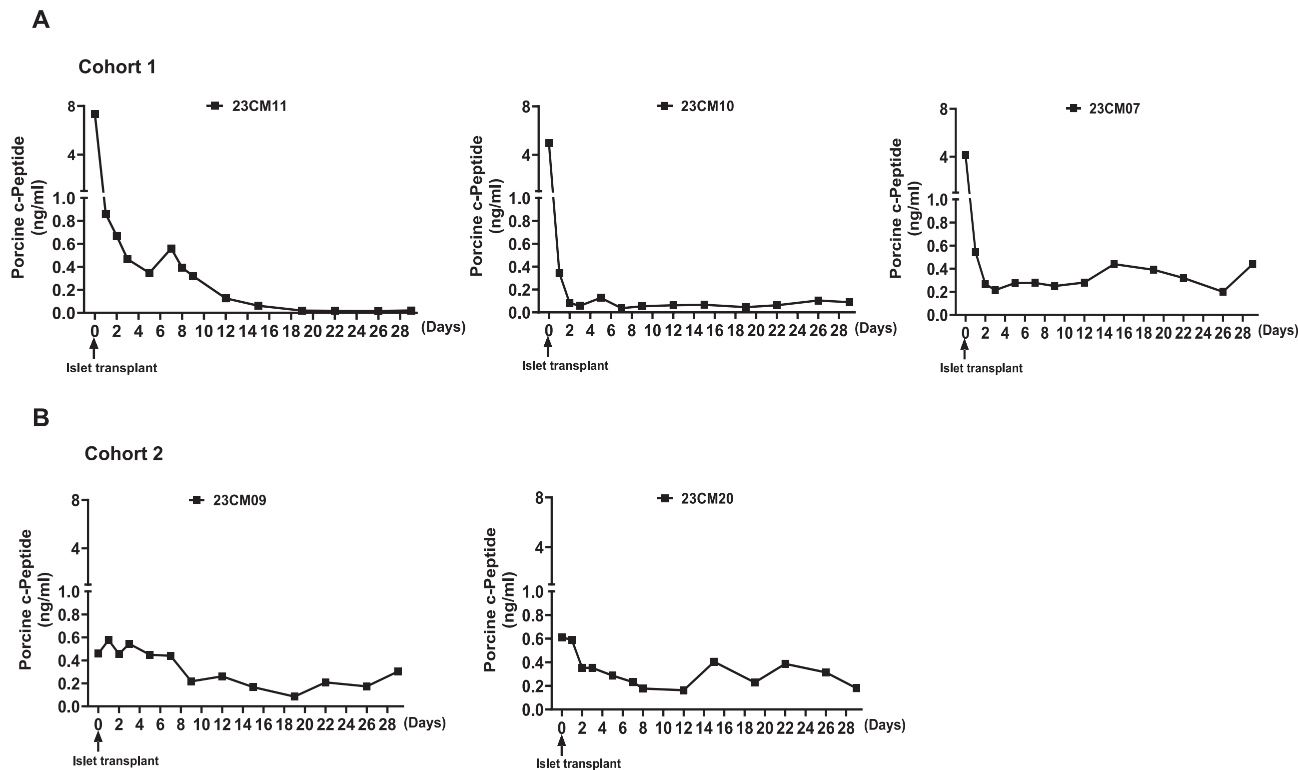

FIGURE 2 | Early islet graft function after QKO porcine islet xenotransplantation. Porcine C-peptide levels were measured in recipient plasma to evaluate early islet graft function. In Cohort 1 (23CM10, 23CM11, 23CM07) (A), C-peptide levels showed a sharp increase at 4 h post-transplantation (> 4 ng/mL) but became undetectable by 2–4 weeks. In contrast, Cohort 2 (23CM09, 23CM20) exhibited an early peak of approximately 0.6 ng/mL at 4 h, with stable levels (0.2–0.4 ng/mL) maintained through 4 weeks posttransplantation (B).

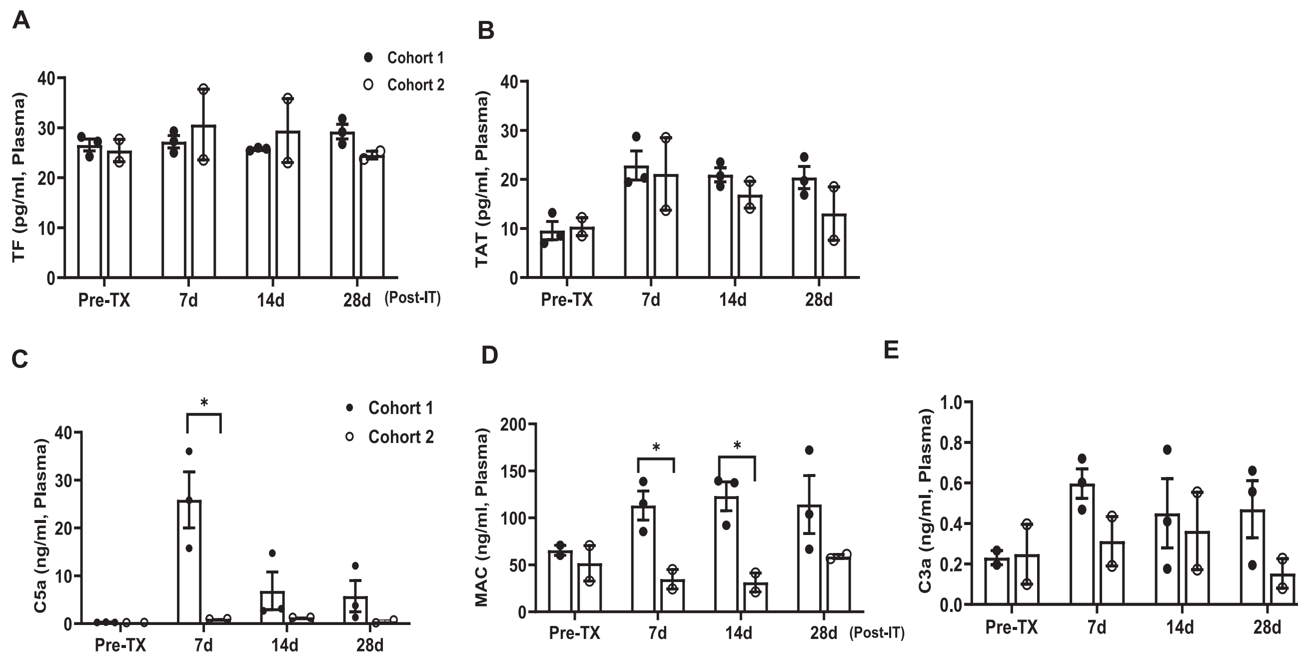

FIGURE 3 | Coagulation factor and complement activation following QKO porcine islet xenotransplantation. Plasma samples were analyzed to assess inflammatory cytokine, coagulation factor, and complement activation after transplantation. Coagulation activation was assessed by quantifying tissue factor (TF) (A) and thrombin–antithrombin complex (TAT) (B) levels. Complement activation was evaluated by measuring C5a (C), membrane attack complex (MAC) (D) and C3a (E) levels, reflecting both upstream (C3a) and downstream (C5a, MAC) complement activity. These data collectively illustrate the contribution of inflammation, coagulation, and complement activation to early xenograft injury. Lines and whiskers in dot plots indicate the mean and SEM. *$p < 0.05$ compared to the cohort 1. Post-IT, Post islet transplant; Pre-TX, Pretransplant (28 days before surgery).

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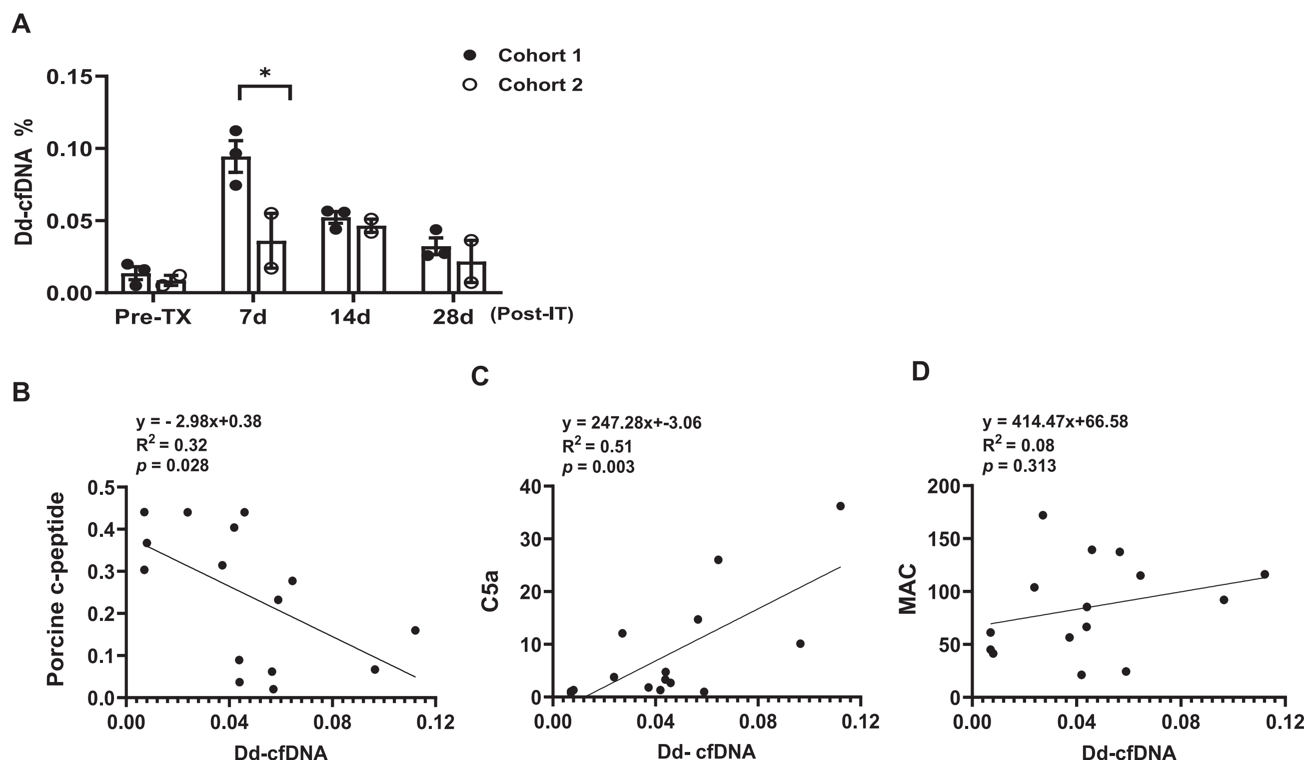

FIGURE 4 | Donor-derived cell-free DNA (dd-cfDNA) and its correlation with graft function and complement activation. (A) dd-cfDNA levels were measured in recipient plasma following QKO porcine islet xenotransplantation. Cohort 1 exhibited a marked peak in dd-cfDNA at postoperative day (POD) 7, whereas Cohort 2 showed lower and more stable levels, indicating improved early graft preservation. dd-cfDNA levels showed a negative correlation with porcine C-peptide levels (B), indicating that lower graft injury was associated with better islet function. In contrast, dd-cfDNA levels showed a positive correlation with C5a (C), whereas no significant correlation was observed with MAC (D). Lines and whiskers in dot plots indicate the mean and SEM. *$p < 0.05$ compared to the cohort 1. Correlation analyses were performed using Pearson's correlation test, with statistical significance defined as $p < 0.05$. Post-IT, Post islet transplant; Pre-TX; Pre-transplant (28 days before surgery).

Early intraportal exposure of porcine islets to recipient blood induces platelet activation, thrombus formation, and complement deposition, which together accelerate IBMIR-mediated early graft injury [8, 9]. Moreover, levels of the coagulation cascade markers, TF and TAT, showed no significant changes in either cohort (Figures 3A and 3B). Such consistency suggests that using QKO pigs as islet donors, together with the baseline immunosuppressive regimen effectively prevented early xenogeneic coagulation activation.

Complement activation is a critical, multifaceted immunological barrier in xenotransplantation that plays a key role in both hyperacute and delayed rejection, for it directly damages the graft endothelium and recruits inflammatory cells [27]. In our study, complement activation markers showed distinct patterns between cohorts. C5a (Figure 3C) and MAC (Figure 3D) levels were significantly increased in Cohort 1 but significantly decreased in Cohort 2. Although C3a levels showed a downward trend in Cohort 2, they did not achieve statistical significance (Figure 4E). The increase in C5a in Cohort 1 reflects a strong pro-inflammatory response and immune cell recruitment, while the elevated MAC indicates direct humoral injury to the graft. Conversely, the significant reduction of both C5a and MAC in Cohort 2 suggests that including complement inhibitors (i.e., CVF and/or Crovalimab) in the regimen suppressed the terminal complement cascade.

DSA levels did not significantly change before or after transplantation in either cohort (Figure S1). Those results indicate that the immunosuppressive regimen, together with the use of QKO pig donors, effectively prevented early DSA induction following islet xenotransplantation.

3.3 | dd-cfDNA as an Early Biomarker of Graft Injury After Porcine Islet Transplantation

Released from apoptotic or necrotic donor cells, dd-cfDNA is a promising noninvasive biomarker for detecting graft injury. It not only reflects graft cell death but elevated levels are associated with active rejection and early graft damage before conventional markers [28, 29]. In our study, dd-cfDNA levels increased after transplantation in both cohorts and continued to rise beyond postoperative day 7. However, the absolute dd-cfDNA level at postoperative day 7 was significantly lower in Cohort 2 than in Cohort 1 (Figure 4A). Its levels were negatively correlated with porcine C-peptide levels (Figure 4B), indicating that as graft injury (i.e., high dd-cfDNA) decreased, islet function (i.e., C-peptide) improved. By contrast, dd-cfDNA levels were positively correlated with C5a (Figure 4C), a potent anaphylatoxin and key marker of complement-mediated inflammation. However, no significant correlation was observed between dd-cfDNA and MAC (Figure 5D). Those results indicate that dd-cfDNA reflects early graft injury and may serve as a useful biomarker for

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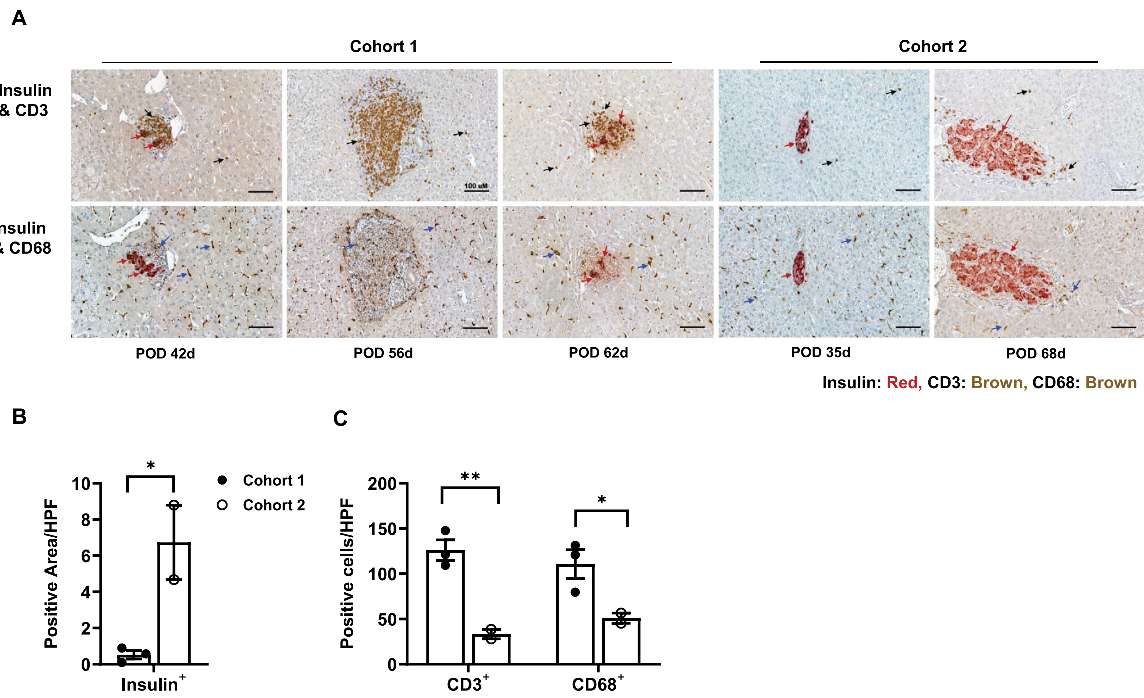

FIGURE 5 | Histological evaluation of islet xenograft mass and immune cells infiltration. Representative biopsy graft sections from Cohort 1 and Cohort 2 were double-stained for insulin (red; red arrows), CD3 (brown; black arrows), and CD68 (brown; blue arrows) at multiple postoperative time points (POD 35–68) using immunohistochemistry (IHC) (A) Quantification was performed using ImageJ: insulin-positive areas (B) were measured, while $CD3^+$ and $CD68^+$ cells were counted (C) (scale bars, 100 µm; magnification, 200×). Cohort 1 showed marked loss of insulin-positive islets with dense $CD3^+$ and $CD68^+$ cell infiltration. In contrast, Cohort 2 exhibited well-preserved insulin-positive islet masses with minimal immune cell infiltration. Lines and whiskers in dot plots indicate the mean and SEM. *$p < 0.05$ compared to the cohort 1. HPF, high-power field.

early graft function and complement-mediated inflammation following porcine islet xenotransplantation.

3.4 | Histological Evaluation of Islet Graft Survival and Immune Cell Infiltration

Histological analysis of liver biopsy sections revealed marked differences in islet graft survival between the two cohorts (Figure 5A). In Cohort 1, insulin-positive islets were largely absent or severely disrupted by postoperative days 42, 56 and 62, accompanied by the dense infiltration of $CD3^+$ T cells and $CD68^+$ macrophages, which indicate immune-mediated graft destruction. By contrast, Cohort 2, which received crovalimab and rituximab, exhibited well-preserved insulin-positive islets with minimal infiltration of $CD3^+$ and $CD68^+$ cells up to postoperative days 35 and 68. Quantitative image analysis confirmed a significantly larger insulin-positive area (Figure 5B) and reduced immune cell infiltration (Figure 5C) in Cohort 2 than in Cohort 1, which suggests improved graft preservation and attenuated inflammation under complement inhibition and B-cell depletion.

4 | Discussion

In our study, we compared two immunosuppressive regimens after QKO (i.e., *GGTA1*, *CMAH*, *B4GALNT2*, and *A3GALT2*) porcine islet xenotransplantation in NHPs. Cohort 1 showed transient C-peptide secretion with strong complement activation (i.e., C3a, C5a, and MAC) and dense immune cell infiltration that led to early graft loss, whereas Cohort 2, treated with crovalimab and rituximab, maintained stable graft function and reduced inflammation. Notably, dd-cfDNA was negatively correlated with porcine C-peptide and positively correlated with C5a, thereby indicating that increased dd-cfDNA reflects complement-mediated islet injury and the loss of graft function. Those findings identify dd-cfDNA as a sensitive, noninvasive marker of early graft injury in islet xenotransplantation.

In our study, recipients received a baseline immunosuppressive regimen consisting of ATG, adalimumab (Humira), tocilizumab, anakinra, tacrolimus, and either MMF or sirolimus, with heparin administered prior to transplantation. That regimen, when combined with QKO donor pigs, depleted $CD3^+$ T lymphocytes (Figure S2) and suppressed DSA formation (Figure S1) and coagulation activation (i.e., TF and TAT), as consistent with published reports [13, 30]. Furthermore, stable levels of TNF-α and IL-6 levels indicated that systemic inflammation was controlled well during the early posttransplant period, with no significant differences observed between the two cohorts (Figure S3). In Cohort 2, adding rituximab achieved effective B-cell depletion and further suppressed humoral immune responses.

Despite the comprehensive baseline immunosuppressive regimen, complement activation remained a major barrier to xenograft survival [31]. Complement-mediated injury has been widely documented across multiple xenograft settings, as documented across various xenograft types, such as corneal [32], islet

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[33], and kidney grafts [34], thereby underscoring its central role in early graft loss, particularly through IBMIR [16, 35]. Although DSA can contribute to complement activation, early complement-mediated injury in islet xenotransplantation often occurs even in the absence of detectable DSA, indicating that innate, rather than adaptive, immune mechanisms primarily initiate early graft damage.

Although the expression of human complement regulatory proteins such as CD46 on Gal-knockout porcine islets can partly attenuate IBMIR by reducing platelet deposition and neutrophil infiltration, it does not completely prevent complement activation [36]. Consistent with that finding, our data showed that even with QKO donor pigs, complement activation markers (i.e., C3a, C5a, and MAC) remained elevated in Cohort 1, thereby indicating that genetic modification alone is insufficient to control complement-mediated injury.

To overcome that challenge, Cohort 2 received crovalimab, a humanized monoclonal antibody targeting C5, along with rituximab for B-cell depletion. Crovalimab [37], was used in Cohort 2 to block terminal complement activation. Crovalimab bound C5 with high affinity, which prevented its cleavage into C5a and C5b, reduced C5a and MAC formation, and mitigated complement-mediated injury. As a result, C5a generation and MAC formation were markedly reduced, which led to the attenuation of IBMIR and improved early graft function, as reflected by stable porcine C-peptide levels and reduced dd-cfDNA release. Even though crovalimab generally inhibits C5a activation and downstream MAC formation, it does not block upstream C3 activation or C3a generation. Therefore, combining crovalimab with a C3 inhibitor [38] may provide broader, more complete protection against complement-mediated xenograft injury and, in turn, enhance graft protection and improve outcomes.

Our correlation analyses additionally demonstrated that dd-cfDNA levels were negatively correlated with porcine C-peptide and positively correlated with C5a, which establishes dd-cfDNA as a dynamic biomarker that reflects the extent of complement-mediated islet injury. Released from damaged or dying islet cells, dd-cfDNA serves as a quantitative indicator of cell injury; it peaked at postoperative day 7 in Cohort 1, coinciding with the rise in C5a and decline in C-peptide, whereas complement inhibition in Cohort 2 attenuated both dd-cfDNA release and C5a activation. Supporting those functional findings, histological evaluation revealed greater residual islet mass and markedly reduced immune infiltration in Cohort 2.

In summary, our study demonstrated that despite QKO genetic modification and comprehensive immunosuppression, complement activation remains a major driver of early xenograft injury. Although crovalimab-mediated C5 blockade effectively mitigated that process, upstream C3 activation persisted, which suggests that the combined inhibition of C3 and C5 may offer relatively comprehensive protection. Moreover, dd-cfDNA seemed to be a reliable, minimally invasive biomarker reflecting complement activation and graft injury and thus provides a valuable tool for early monitoring and timely intervention to improve long-term islet xenograft survival.

Author Contributions

Ji-Jing Yan and Jong-Min Kim equally contributed to this work. Ji-Jing Yan and Jong-Min Kim participated in data curation, methodology, formal analysis, and original draft manuscript; Sang-Ik Cho, Kyungmin Kwak, Hyunil Kim participated in data curation and methodology; Eun-Jee Oh and Hyori Kim participated in conceptualization and formal analysis; Chung-Gyu Park., Jong Cheol Jeong and Beom Seok Kim participated in conceptualization, formal analysis, supervision, and editing manuscript. All authors approved the final version of the manuscript. Crovalimab was synthesized and supplied by Hyori Kim.

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Ethics Statement

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Biomedical Research Institute at Seoul National University Hospital (IACUC No. 23-0025-S1A3). All procedures involving the handling and care of animals were performed in accordance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the US National Institutes of Health (NIH Publication No. 86-23, revised 2011).

Consent

Not applicable. This study did not involve human participants or patient data.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Figure 1: Peripheral blood lymphocytes following QKO porcine islet xenotransplantation. **Supporting Figure 2:** Cytokine levels on QKO porcine islet xenotransplantation. **Supporting Figure 3:** Donor-specific antibody (DSA) response following QKO porcine islet xenotransplantation. **Supporting Table 1:** Antibodies information.

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