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ORIGINAL ARTICLE

Long-term control of diabetes by tofacitinib-based immunosuppressive regimen after allo islet transplantation in diabetic rhesus monkeys that rejected previously transplanted porcine islets

Jong-Min Kim[1,2,3,4,5] | Seong-Jun Kang[1,2,6] | So-Hee Hong[7] | Hyunwoo Chung[1,2,6] | Jun-Seop Shin[1,3,4] | Byoung-Hoon Min[1,3,4] | Hyun Je Kim[1,2,4,6] | Jongwon Ha[8] | Chung-Gyu Park[1,2,3,4,6]

[1]Xenotransplantation Research Center, Seoul National University, College of Medicine, Seoul, South Korea

[2]Department of Microbiology and Immunology, Seoul National University, College of Medicine, Seoul, South Korea

[3]Transplantation Research Institute, Seoul National University Medical Research Center, Seoul National University, College of Medicine, Seoul, South Korea

[4]Cancer Research Institute, Seoul National University, College of Medicine, Seoul, South Korea

[5]Department of Animal Health, Cheongju University College of Health and Medical Sciences, Cheongju, South Korea

[6]Department of Biomedical Sciences, Seoul National University, College of Medicine, Seoul, South Korea

[7]Department of Microbiology, College of Medicine, Ewha Womans University, Seoul, Republic of Korea

[8]Department of Surgery, Seoul National University College of Medicine, Seoul, South Korea

Correspondence
Chung-Gyu Park, Department of Microbiology and Immunology, Xenotransplantation Research Center, Seoul National University College of Medicine. 103 Daehak-ro, Jongno-gu, Seoul 03080, South Korea.
Email: chgpark@snu.ac.kr

Abstract

Porcine islet xenotransplantation has been highlighted as an alternative to allo islet transplantation. Despite the remarkable progress that has been made in porcine-islet pre-clinical studies in nonhuman primates, immunological tolerance to porcine islets has not been achieved to date. Therefore, allo islet transplantation could be required after the failure of porcine islet xenotransplantation. Here, we report the long-term control of diabetes by allogeneic pancreatic islet transplantation in diabetic rhesus monkeys that rejected previously transplanted porcine islets. Four diabetic male rhesus monkeys received the porcine islets and then allo islets (5700—19 000 IEQ/kg) were re-transplanted for a short or long period after the first xeno islet rejection. The recipient monkeys were treated with an immunosuppressive regimen consisting of ATG, humira, and anakinra for induction, and sirolimus and tofacitinib for maintenance therapy. The graft survival days of allo islets in these monkeys were >440, 395, >273, and 127, respectively, similar to that in allo islet transplanted cynomolgus monkeys that received the same immunosuppressive regimen without xeno sensitization. Taken together, it is likely that prior islet xenotransplantation does not affect the survival of subsequent allo islets under clinically applicable immunosuppressants.

Abbreviations: AST, arginine stimulation test; ATG, anti-thymoglobulin; DPT, day post transplantation; IVGTT, Intravenous glucose tolerance test; IXA, international xenotransplantation association; NHP, Nonhuman primate; PERV, porcine endogenous retrovirus; RhCMV, rhesus monkey cytomegalovirus; STZ, Streptozotocin; TNF, tumor necrosis factor.

Jong-Min Kim, Seong-Jun Kang, and So-Hee Hong contributed equally to this work.

KEYWORDS
allo islet transplantation, nonhuman primate, re-transplantation, xeno islet transplantation

1 | INTRODUCTION

Progress in islet isolation, islet culture, and peri-transplant management techniques has led to allo islet transplantation becoming an established approach to cure type I diabetes mellitus.[1] However, due to donor organ shortage, porcine islet xenotransplantation is considered an alternative option.[2] Many groups, including ours, have shown the improved graft survival of porcine islets in nonhuman primate preclinical studies[3–5] and have tried to fulfill the consensus statement on the requirement for clinical trials of porcine islet transplantation of the International Xenotransplantation Association (IXA) to guarantee the safe and efficacious pig-to-NHP islet xenotransplantation in the clinic. According to this consensus, a preclinical trial would be considered a success if four of six diabetic NHPs receiving porcine islets maintained normoglycemia for at least 6 months without any or with a significantly reduced level of exogenous insulin therapy.[6] A recent study performed by our group showed that long-term porcine islet graft survival in three of seven diabetic NHPs for 6 months without severe adverse effects could be achieved by clinically available immunosuppressive agents.[7] Thus, the possibility of the clinical application of porcine islet transplantation is growing. Notwithstanding this achievement, tolerance to the porcine islet graft has not been established yet and engrafted porcine islets are rejected gradually over time. Thus, additional xeno or allo islet transplantation might be needed after the rejection of the first xenotransplantation in the clinic, and the development of an effective immunosuppressive regimen that can control the second xeno or allo islet transplantation is required.

In a previous study, our group demonstrated that subsequent allo islet transplantation after porcine islet sensitization restored normoglycemia under a proper immunosuppressive regimen in a murine model.[8] To expand upon this earlier work, we initiated an exploratory study conducted within a limited cohort involving the re-transplantation of allo islets into diabetic rhesus monkeys who had already undergone rejection of porcine islet xenotransplantation. For this study, we selected tofacitinib as the maintenance drug. Tofacitinib, a JAK inhibitor, can control the cytokine induced signaling transduction pathway that affects various cellular processes. It has been shown to inhibit the functions of T, B, and NK cells by inhibiting IL-2, IL-4, IL-17, and IL-15 signaling pathways.[9] Thus, tofacitinib used as an immunosuppressive drug, such as rheumatoid arthritis, and used in renal transplantation.[10] In fact, our previous study demonstrated that a JAK3-based immunosuppressive regimen prolonged the survival of allo islets in cynomolgus monkeys.[11] Furthermore, we found that tofacitinib prevented the early loss of islet mass by oxidative stress in a murine syngeneic islet transplantation model and increased the viability of islets under oxidative stress (unpublished data). Similar to the oxidative stress-reducing effects of tofacitinib, Yang et al. showed that tofacitinib protected intestinal epithelial cells against oxygen-glucose deprivation/reoxygenation injury by inhibiting the JAK/STAT3 signaling pathway.[12] Also, it is known that calcineurin inhibitors such as cyclosporine and tacrolimus have cytotoxic effects on pancreatic beta cells. Thus, we believe that the protective effects of tofacitinib on islets would be an advantage in islet transplantation and tested whether a tofacitinib based clinically immunosuppressive regimen could induce the long-term survival of transplanted allo islets.

2 | MATERIALS AND METHODS

2.1 | Animals

A total of nine rhesus monkeys (*Macaca mulatta*) were used in this study. Four male rhesus monkeys, 4–11 years of age, were used as recipients. They were transplanted with porcine islets 26–1534 days before allo islet transplantation and received various immunosuppressive regimens (Table 1). Exogenous insulin treatment was performed until allo islets were transplanted. Five female rhesus monkeys were used as islet donors. The median age of the five donor rhesus monkeys was 56 months (range, 48–67). After being imported from China, a quarantine process of 1 month concluded that all subjects were in good general condition. Then, the monkeys were acclimated to our animal facility for at least 6 months. All procedures that affected the handling and care of animals were in compliance 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). The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Biomedical Research Institute at the Seoul National University Hospital (AAALAC accredited facility; IACUC number: 15–0297).

2.2 | Induction of diabetes, porcine islet transplantation, and exogenous insulin treatment of NHPs

A detailed description of the procedures used for the induction of diabetes, porcine islet transplantation, and exogenous insulin treatment of NHPs is provided in the Supplementary Materials and Methods.

Four recipients were previously transplanted with porcine islets (81 000—100 000 IEQ/kg) with immunosuppressive drugs (Table 1). There were 11–534 days after the rejection of porcine islets until allo islet transplantation. The recipients who were the absence of porcine islets graft function were managed with exogenous insulin to prevent hyperglycemia until allo islet transplantation.

TABLE 1 History of porcine islet transplanted rhesus monkeys.

Recipient	History	Transplanted porcine islet mass (IEQ/kg)	Graft survival day	Days from porcine islet rejection before allo-islet transplantation
R051	Anti-CD 154 mAb switched to anti-CD 40 mAb based immunosuppressive regimen	81 000	1000	534
R144	Anti-CD 40 based immunosuppressive regimen + tacrolimus + tocilizumab	100 000	22	488
R172	ATG, IVIg, humira, anakinra, tocilizumab, tofacitinib, sirolimus or Mp/d + Bortezomib	100 000	15	11
R173	ATG, IVIg, humira, anakinra, tocilizumab, tofacitinib, sirolimus or Mp/d + Bortezomib	100 000	30	14

Note: Results of R051 were published in (American Journal of Transplantation 2015; 15: 2837–2850) and (Xenotransplantation 2016: 23: 300–309). Result of R144 was published in (2018 Jan;25(1):10.1111/xen.12374.

TABLE 2 Quality of isolated islets, anti-donor stimulation index, and graft survival in intrahepatic allo islet transplanted diabetic rhesus monkeys.

Recipient	Donor	IS regimen	Infused allo-islet mass (IEQ/kg)	Purity (%)	Frag-mentation index	Anti-donor MLC, Stimulation index (-fold increase)	POD C-peptide (>1.0 ng/mL)	Days off insulin	Days C-peptide positive
R051	R196	ATG, Humira, Sirolimus, Tofacitinib	14 000	85	1.44	0.94	0	3	41
R051 (D 41: Re TPL)	R218	Basiliximab, Humira, Anakinra, Sirolimus, Tofacitinib	13 700	82	2.00	0.99	>440	>440	>440
R144	R206	ATG, Humira, Anakinra, Sirolimus, Tofacitinib	7000	69	0.9	NE	81	2	81
R144 (D 82: Re TPL)	R218	Basiliximab, Humira, Anakinra, Sirolimus, Tofacitinib	7000	82	2.00	1.45	310	238	395
R172	R215	ATG, Humira, Anakinra, Sirolimus, Tofacitinib	19 000	83	3.36	NE	>273	>273	>273
R173	R215	ATG, Humira, Anakinra, Sirolimus, Tofacitinib	18 000	83	3.36	NE	47	53	91
R173 (D 91: Re TPL)	R198	Basiliximab, Humira, Anakinra, Sirolimus, Tofacitinib	5700	83	3.36	1.96	0	4	127

Abbreviation: NE: not exam.

2.3 | Islet isolation from rhesus monkeys

A detailed description of the procedures used for islet isolation from rhesus monkeys is provided in the Supplementary Materials and Methods.

The purity of isolated islets (n = 5) was 80.4 ± 6.5% and the fragmentation index was 2.2 ± 1.1 (Table 2).

2.4 | Islet transplantation into NHPs

All monkeys were fasted for 12 h before surgery. A laparotomy was performed, and a jejunal mesenteric arch was exposed to infuse the islets. A 22-gauge catheter was inserted into a jejunal vein. Rhesus islets for transplantation (5700—19 000 IEQ/kg) with 100 IU/kg heparin were infused under gravity pressure over 8–12 min. After infusion, the vessel was ligated with a 5-0 Prolene suture. Ketamine (50 μg/kg/min) and lidocaine (0.6 mg/kg/h) were infused continuously for 3 days. Meloxicam (0.1 mg/kg, i.v., s.i.d.) was injected for 3 days, and butorphanol (0.05 mg/kg, i.v.) for postoperative analgesia was injected when necessary. After surgery, the tether system was applied for continuous fluid therapy and infusion of low-dose dextrose, if necessary.

2.5 | Immunosuppressive therapy for allo islet transplantation

Immunosuppressive regimens consisted mainly of ATG (first transplantation) or basiliximab (re-transplantation), adalimumab, and anakinra (except R051 in the first transplantation) for induction, and

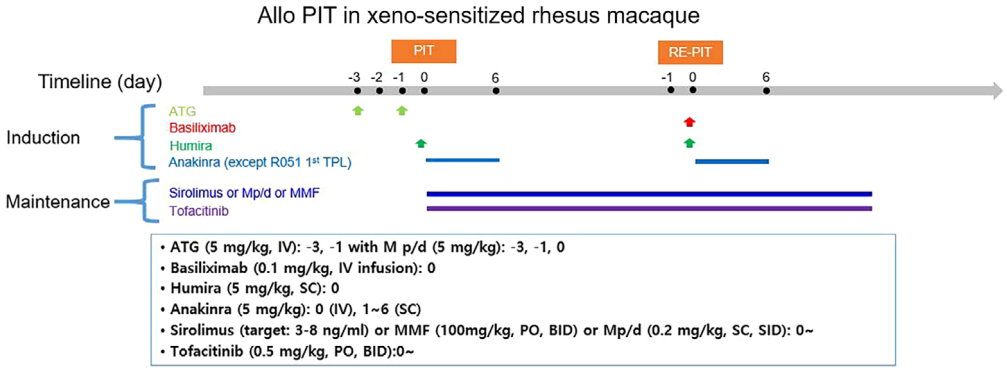

FIGURE 1 Immunosuppressive protocol consisting of clinically-applicable agents for allo islet transplantation in a porcine islet sensitized NHP model.

sirolimus and tofacitinib for maintenance therapy (Figure 1). This immunosuppressive regimen was previously evaluated and reported by our group for diabetic cynomolgus monkeys receiving an allo islet transplantation.[11] When the first islet graft was partially rejected as evidenced by low-level monkey C-peptide and hyperglycemia, a second islet transplantation was performed as described above. Anti-thymocyte globulin (ATG; 5 mg/kg, i.v., −3 and −1 days; Thymoglobulin, Genzyme, Cambridge, MA), basiliximab (0.3 mg/kg, i.v., 2 h before islet infusion; Simulect Inj. Norvartis Pharmaceuticals Corp., Basel, Switzerland), adalimumab (5 mg/kg, s.c., 2 h before islet infusion; Humira, Abbott Laboratories Ltd., Queenborough, UK), anakinra (5 mg/kg, i.v. on day 0 and s.c. on days 1–6; Kineret, Amgen, Seoul, Korea), sirolimus (target trough concentration: 5–8 ng/mL, p.o., 0 day ~; Rapamune, Astellas Pharma Korea, Seoul, Korea), and tofacitinib (0.75 mg/kg, p.o., b.i.d., 0 day ~; Xeljanz, Pfizer Korea, Seoul, Korea) were administered. Sirolimus was also administered, and in cases of drug-related complications, it was switched to methylprednisolone (0.3 mg/kg, sc, s.i.d.) or mycophenolate mofetil (20 mg/kg, p.o., b.i.d.; Cellcept, Korea Roche, Seoul, Korea) (See Table 3). The target trough levels (3–8 ng/mL) were sustained by adjusting the dosages of sirolimus (0.08–0.3 mg/kg/d) after confirming the serum concentration by liquid chromatography-tandem mass spectrometry.

TABLE 3 Summary of used immunosuppressive agents and their individual targets within the immune responses.

Immunosuppressive agents	Target
Anti-CD154 or CD40 antibody	cellular and humoral adaptive immunity
ATG	T cell depletion
Basiliximab	T cell anergy
Bortezomib	Humoral adaptive immunity
Tacrolimus	T cell suppression
Sirolimus	T cell suppression
Tofacitinib	Inhibition of T cells, B cells, NK cells, memory T cells through IL-2, IL-4, IL7, IL-15 signaling pathway
Humira	TNFa blocker
Anakinra	IL-1b blocker
Tocilizumab	IL-6 blocker
IVIg	Modulation of B and T cells, phagocytosis, complement activity, cytokine production, and the properties of dendritic cells
Mp/d	Anti-inflammatory

2.6 | Monkey C-peptide measurements

To measure serum C-peptide concentrations, blood samples were collected in a serum separating tube. Blood samples were centrifuged at 2990 *g* for 20 min at 4°C, and the separated serum was frozen and stored at −80°C until further use. Monkey C-peptide concentrations were determined by an immunoradiometric assay (C-peptide IRMA Kit, Immunotech, Czech) according to the manufacturer's instructions.

2.7 | Intravenous glucose tolerance test (IVGTT)

To monitor islet graft function during follow-up, an IVGTT was performed. In brief, after overnight fasting without insulin, 0.5 g/kg of a 50% dextrose solution was added to the same volume of normal saline and infused i.v. over 1 min. Blood glucose levels were measured in monkeys before and at 2, 5, 15, 30, 60, 90, and 120 min after infusion. C-peptide levels were measured at the same time intervals. The glucose disappearance rate (KG) represents the log-linear (ln) decline in glucose levels between 5 and 30 min after glucose infusion, and was calculated using the following formula: KG = ln (glucose level at 5 min)−ln (glucose level at 30 min)/25 × 100.

2.8 | Flow cytometry

A detailed description of the procedures used for flow cytometry is provided in the Supplementary Materials and Methods.

2.9 | ELISPOT

The numbers of IFN-γ-secreting cells, specific to pig PBMCs or allogenic monkey PBMCs were measured using an ELISpot kit (Mabtech, Nacka Strand, Sweden) as previously described.[7] The resulting spots were enumerated by a computer-assisted ELISPOT Reader System (AID, Strassberg, Germany).

2.10 | Anti-donor mixed leukocyte culture (MLC)

To evaluate the alloreactive capacity of donor lymphocytes towards recipient cells, a mixed lymphocyte culture (MLC) was performed as described previously.[11] Recipient PBMCs were used as responders against γ-irradiated (3000 rad) donor PBMC in a one-way mixed leukocyte culture (MLC). Culture media consisted of RPMI 1640 medium supplemented with 10% normal bovine serum, antibiotics, Hepes, sodium pyruvate, and L-glutamine. Responder cells were labeled with CFSE and harvested and analyzed on day 6 by CFSE dilution assay using FACS. Data are expressed as the stimulation index: anti-donor CFSE dilution percentage divided by that of the responder only (Table 2).

2.11 | Biopsy and immunohistochemistry

A detailed description of the procedures used for biopsy and immunohistochemistry is provided in the Supplementary Materials and Methods.

2.12 | Rhesus monkey cytomegalovirus (RhCMV) copy numbers and antibody levels

A detailed description of the procedures used to determine the RhCMV copy numbers and antibody levels are provided in the Supplementary Materials and Methods.

2.13 | Statistical analysis

We analyzed alterations in absolute cell numbers from baseline at specific time points during the post-transplant period and compared them with a group of historical comparators ($n = 5$) who had not been exposed to a porcine donor. To perform this analysis, we employed the Kruskal–Wallis test, followed by Dunn's multiple comparisons between the designated time points. The spot numbers in the ELISPOT assay were compared among groups, and statistical differences were assessed using the ANOVA test. The changes in alloreactivity across all four animals following exposure to a porcine donor were compared using Student's *t*-test. Differences were considered statistically significant when the *p*-value was less than .05 (The statistical software used was GRAPHPAD PRISM5; GraphPad Software, La Jolla, CA, USA).

3 | RESULTS

3.1 | Allo islet transplantation after xeno islet rejection restored normoglycemia

The primary goal of this study was to test whether a tofacinib-based immunosuppressive regimen could restore and maintain normoglycemia for a long period in diabetic NHPs that received allo islet transplantation after xeno islet rejection. To this end, four diabetic rhesus monkeys that had experienced rejection of previous porcine islet xenotransplantation were used as allo islet recipients (Table 1). In this study, we used a JAK3 inhibitor-based immunosuppressant regimen as a maintenance therapy because it significantly prolonged the survival of transplanted allo islets in cynomolgus monkeys.[11]

14 000 IEQ/kg was transplanted on R051 in the first allo islet transplantation, which resulted in a decrease of approximately 40% in exogenous insulin compared to before transplantation. On D41, allo islet retransplantation of 13 700 IEQ/kg was performed, which showed exogenous insulin-free results for more than 400 days, but fasting blood glucose level was slightly elevated after D400 until elective termination. R144 was transplanted 7000 IEQ/kg in the first allo islet transplantation, which resulted in a decrease of approximately 80% in exogenous insulin compared to before transplantation. On D82, allo islet retransplantation of 7000 IEQ/kg was performed, which showed insulin-free results for 150 days, and the graft gradually deteriorated, and insulin requirements increased. Elective termination was performed on day 318. First allo islet transplantation of 18 000 IEQ/kg was conducted on R173, which showed insulin-free results for 53 days, and the graft deteriorated gradually, leading to increased insulin requirements and undetectable levels of monkey C-peptide. On D91, allo islet retransplantation of 5700 IEQ/kg was performed, but insulin-free results were not achieved, and only low levels of monkey C-peptide were detected. Elective termination was performed on D137.

Although three monkeys were re-transplanted with another batch of allo islets after the first batch of allo islet grafts were partially rejected as evidenced by a low level of monkey C-peptide and hyperglycemia, they restored and maintained normoglycemia or monkey C-peptide positive after the second infusion of allo islets. The monkey C-peptide levels were positive for >440, 395, and 127 days in R051, R144, and R173, respectively (Table 2 and Figure 2A). Of note, R172 maintained normoglycemia for more than 273 days without an additional infusion of allo islets (Figure 2A). They showed normal ranges of HbA1c levels while maintaining normoglycemia (Figure 2B). The IVGTT, which was performed at regular intervals (1–2 months), and kinetics of porcine C-peptide levels during the IVGTT, revealed that the transplanted islets were functional (Figure 3). It seems that the early deteriorated functions of first allo islet transplantation might have occurred because of the insufficient control of cytokine mediated inflammation because anti-inflammatory reagents were not used in the regimen for R051, and the relatively low purity (69%) and low fragmentation index (0.9) in R144 (Table 2). After this early deterioration in the first allo islet transplantation in R051, we added anakinra to the immunosuppressive regimen to block IL-1β in the other recipients.

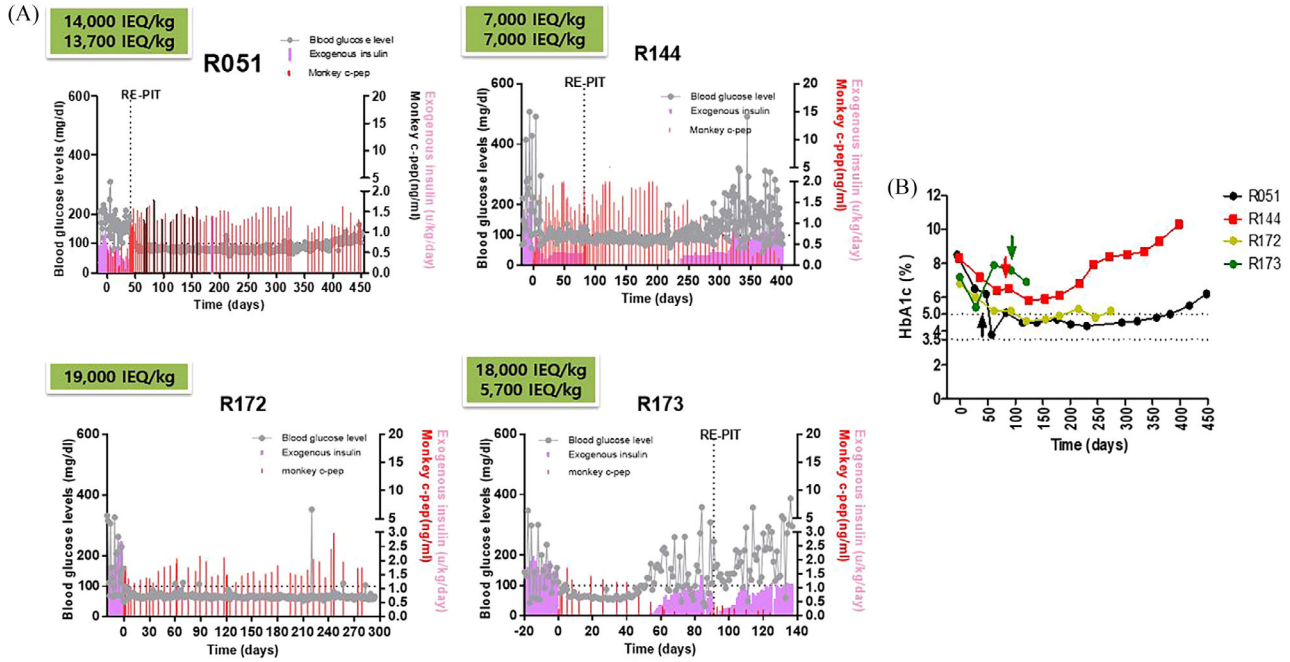

FIGURE 2 Blood glucose levels (BGL) and HbA1c levels at the first and second allo islet transplantations in porcine islet-sensitized diabetic monkeys. (A) Fasting blood glucose levels and monkey C-peptide levels in allo islet transplanted monkeys were measured. Gray line = fasting BGL; red bar = monkey C-peptide; pink bar = exogenous insulin administered. RE-PIT means re-transplantation of allo islet. (B) HbA1c levels were measured at approximately 1-month intervals. The normal range of HbA1c in macaques is 3.5%–5.0%. Arrows indicate the day of re-transplantation of allo islets.

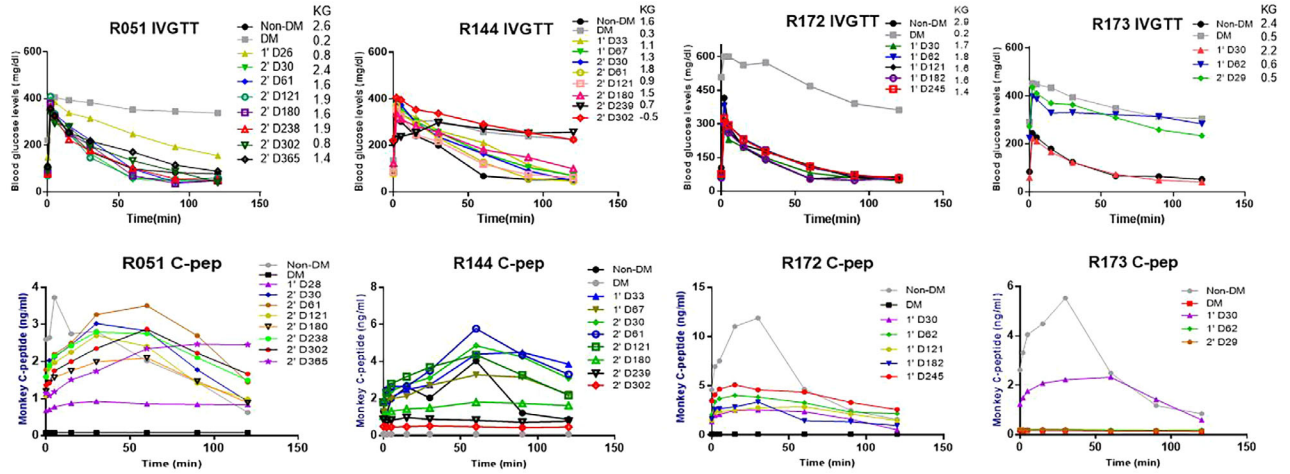

FIGURE 3 Blood glucose and monkey C-peptide responses during the intravenous glucose tolerance test. IVGTTs were performed in individual allo islet-transplanted monkeys at the indicated time points. The glucose clearance rate (KG) represented at each time point demonstrated that each monkey had disturbed glucose disposal capacity (KG; 1–2) although they were normoglycemic in fasting and post-prandial. Secreted monkey C-peptide in response to glucose injection was detected in all monkeys.

R172 and R173 have the same immunosuppressive regimen, but R173 experienced a faster rejection response and underwent two transplant procedures. Since both R172 and R173 received an allo islet from the same donor, the quality of the pancreas cannot be the reason for the difference in outcomes. However, the cellular reactivity of R173 was more pronounced than that of R172 after allo#xen12850-fig-0004.fig transplantation, suggesting that cellular immune rejection may be the cause (Figure 5). We believe that the early deteriorated function of the second allo islet transplantation in R173 might have resulted from the relatively low infused islet mass (5700 IEQ/kg) and higher anti-donor MLC response (1.96) compared to the other pairs (0.94, 0.99, 1.45) (Table 2).

In a previous study, three cynomolgus monkeys that received two separate infusions of allo islets, maintained normoglycemia for 98, >330, and >128 days under a JAK3 inhibitor-based regimen.[11] Therefore, it seems that the JAK3 inhibitor-based regimen could also induce

the long-term survival of allo islets in recipient monkeys that had already experienced xeno islet rejection.

3.2 | Changes in the lymphocyte populations of recipient monkeys after allo islet transplantation

Next, we measured the absolute T, B, and NK cell numbers after allo islet transplantation by flow cytometry. Between 2 and 5 days before allo islet transplantation, the average peripheral $CD4^+$ and $CD8^+$ T cell counts were 841 ± 307/μL and 820 ± 211/μL, respectively (Figure 4). These numbers are comparable to the average $CD4^+$ and $CD8^+$ cell numbers before porcine islet transplantation. Thus, xeno islet rejection did not significantly affect the numbers of T cells in recipients. Two ATG injections on −3 and −1 days post transplantation (DPT) significantly decreased the number of $CD4^+$ and $CD8^+$ T cells transiently, although the absolute numbers of $CD20^+$ B cells were not affected by the ATG treatment (Figure 4). The numbers of T cells in all recipients returned to their previous levels at 6.75 ± 2.7 days post-transplantation. Consistent with previous studies, the absolute numbers of $CD4^+$ or $CD8^+$ T cells did not reflect the changes in blood glucose levels (Figures 2A and 4).

3.3 | Monitoring of cell-mediated immune responses to xeno or allo donor antigens

Next, we checked the cellular responses of recipients specific for xeno or allo antigens by ELISPOT. Before xeno islet transplantation, 593 ± 59, 245 ± 72, and 223 ± 32 IFN-γ producing cells per 5×10^5 responder cells were detected after xenoantigen stimulation in R051, R172, and R173, respectively (Figure 4). The numbers of alloantigen reactive IFN-γ producing cells were lower than the numbers of xenoantigen reactive cells in R051 (spot number: 243 ± 30) and R172 (spot number: 46 ± 11) but similar numbers were detected in R173 (spot number: 219 ± 38). Without stimulation, 45–150 IFN-γ-producing cells were detected in the responder cells of recipients (R051: 45 ± 32, R173: 150 ± 13). Unfortunately, we could not perform the ELISPOT assay in R144 due to the lack of samples at that time.

After xeno islet transplantation, the numbers of IFN-γ producing cells in response to the xenoantigen were decreased compared with before transplantation (spot number: R051: 228 ± 11, R144: 254 ± 40, R172:87 ± 11, R173: 144 ± 8). Similarly, the numbers of IFN-γ producing cells responding to alloantigens were also decreased compared with those before xeno islet transplantation (spot number; R051: 141 ± 18, R144; 119 ± 16 R172:17 ± 3, R173: 148 ± 23). Without stimulation, the numbers of IFN-γ-producing cells of R051, R144, R172, and R173 were 45 ± 11, 51 ± 16, 35 ± 18, and 130 ± 3, respectively.

Thus, it seems that the cellular immune responses to xeno or alloantigens were controlled by our immunosuppressants at the early phase of xeno islet transplantation. However, after xeno islet rejection, the numbers of IFN-γ producing cells to the xenoantigen were increased in R144 (spot number; 635 ± 27), R172 (spot number; 395 ± 289), and R173 (spot number; 295 ± 10), but not R051 (spot number; 162 ± 7) (Figure 5). Although alloantigen reactive IFN-γ producing cells remained low in R051 (spot number; 90 ± 22), R172 (spot number; 6 ± 4), and R173 (spot number; 5 ± 8), alloantigen reactive IFN-γ producing cells were increased in R144 (566 ± 15) after xeno islet rejection. The numbers of IFN-γ-producing cells in R051, R144, and R172, without stimulation were 82 ± 4, 75 ± 25, and 63 ± 40, respectively.

After allo islet transplantation, the numbers of IFN-γ producing cells specific for alloantigens remained lower in R172 (spot number; 23 ± 7) and R173 (spot number; 63 ± 16), which indicated the long-term survival of the first allo islet transplantation. In contrast, relatively increased spot numbers were observed in R144 (381 ± 15) and R051 (242 ± 26).

Furthermore, numbers of xenoantigen-reactive IFN-γ-producing cells were higher than those of allo antigen-reactive cells in all recipients (spot number; R051; 476 ± 88, R144; 335 ± 46, R172; 147 ± 14, R173; 160 ± 36) after allo islet transplantation. However, compared with those after xeno islet rejection, numbers of xeno antigen-specific IFN-γ producing cells were decreased after allo islet transplantation in R172, R144, and R173. Without stimulation, the numbers of IFN-γ-producing cells in R051, R144, R172, and R173 were 144 ± 16, 76 ± 16, 13 ± 1, and 91 ± 3, respectively (Figure 5).

3.4 | Immunohistochemical staining of liver biopsies

To identify the graft infiltrating cells and integrity of engrafted allo islets, we performed IHC for markers of T cells (CD3), B cells (CD20), and macrophages (CD68). Considerable numbers of allo islets were observed in the liver samples from R051 (DPT 287) and R172 (DPT 86). However, the majority of allo islets were destroyed in R144 (DPT 328) and R173 (DPT 86). The main infiltrating cells after allo islet rejection were $CD3^+$ T cells in R144 and R173 although B cells and macrophages were also detected. Almost intact allo islets were detected in R172 (DPT 86) and islets from R051 exhibited some immune cell infiltration including T cells and macrophages (Figure 6A, B).

3.5 | General condition of recipient monkeys after allo islet transplantation

Physical conditions of recipients are critical for evaluating transplantation outcomes. Vital signs such as body temperature, heart rate, respiration, appetite, and physical activity were normal after allo islet transplantation and during treatment with the immunosuppressants (data not shown). All monkeys maintained an average body weight without significant reduction after allo islet transplantation (Figure 7). Other adverse events, such as anorexia, diarrhea, and constipation, were transiently observed in some recipient monkeys. Blood examination revealed intermittent neutrophilia and increased CRP levels that

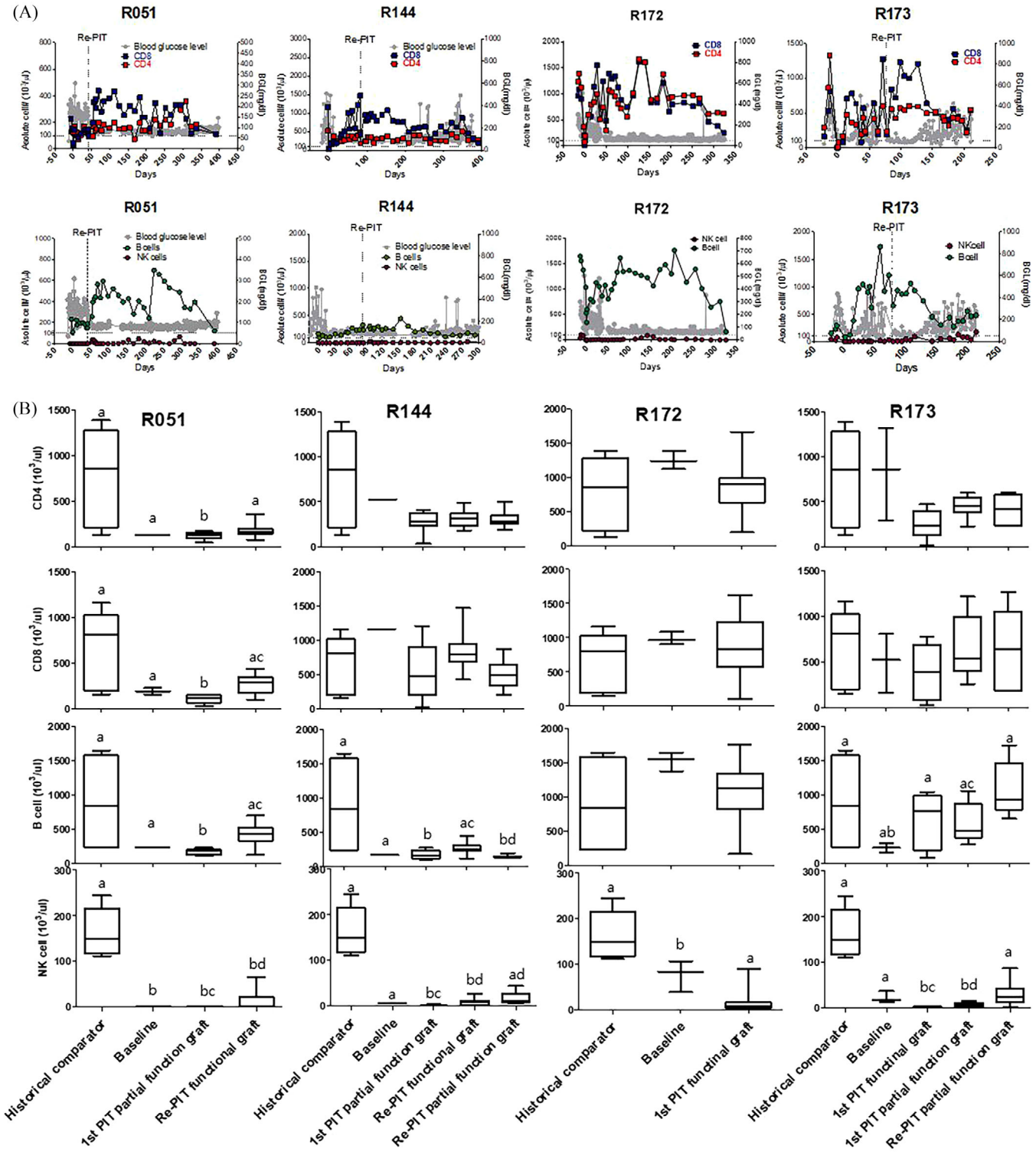

FIGURE 4 Monitoring of the absolute numbers of lymphocytes in the peripheral blood of the recipient monkeys. (A) The absolute numbers of peripheral CD4+ and CD8+ T cells, CD20+ B cells, and NK cells were assessed at the indicated time-points by flow cytometric analysis. The levels of NK cells declined due to tofacitinib after allo-islet transplantation. (B) Changes in absolute cell numbers from baseline at the selected timepoints during the post-transplant period, comparing them with historical comparators ($n = 5$) who had not been exposed to a porcine donor. Data were analyzed using Kruskal–Wallis test with Dunn's multiple comparisons between the selected timepoints. Different letters were used to represent statistically significant differences.

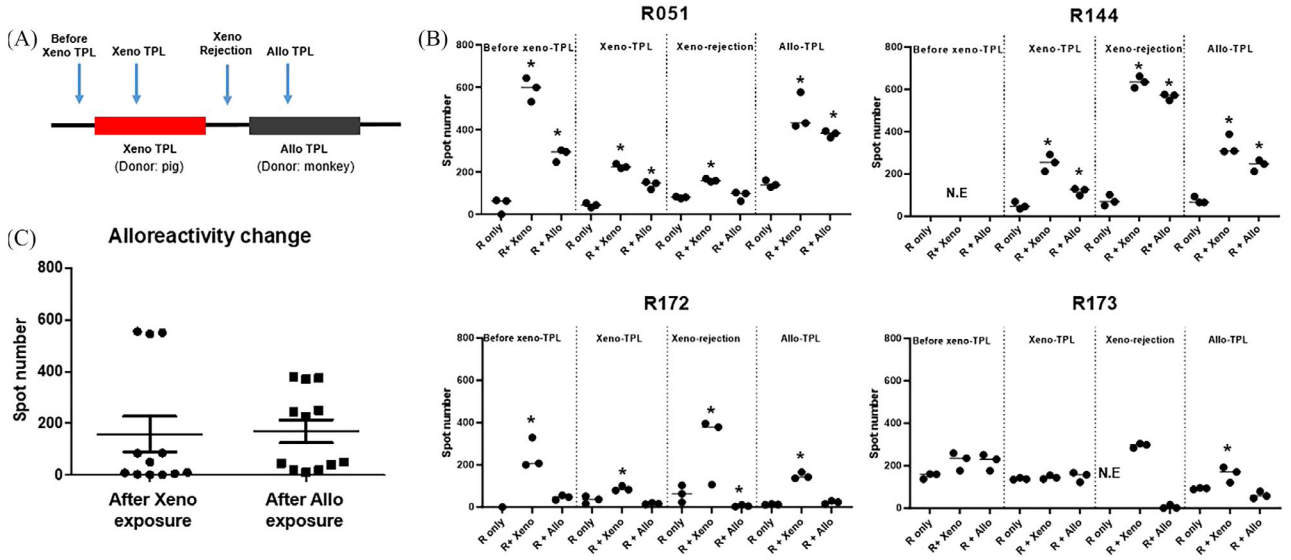

FIGURE 5 Cellular reactivity of recipient monkeys toward donor antigens. PBMCs from recipients were prepared before and 2 weeks after porcine islet TPL and allo islet transplantation. 5×10^5 responder cells were stimulated with irradiated (30 Gy) pig PBMCs (for xeno) or monkey PBMCs (for allo). After 24 h of culture, IFN-γ producing cells were estimated by ELISPOT assay. (A) Sample collection points for ELISPOT assay. (B) Cellular reactivity of each recipients to Xeno or Allo antigen. The spot number in ELISPOT assay were compared among groups, and statistical differences were assessed using the ANOVA test. C: The changes in alloreactivity across all four animals following exposure to a porcine donor. *p*-values are significant according to the Student's *t*-test; $^*p < .05$. NE: not examined.

were normalized with antibiotic treatment (data not shown). These results indicate that our immunosuppressive regimen is safe and do not induce severe adverse effects due to the excessive immunosuppression. We also checked RhCMV reactivation in recipient monkeys because CMV reactivation can occur after organ transplantation due to severe immunosuppression. We performed a real-time PCR assay to confirm the Rh CMV reactivation every month during immunosuppression therapy. RhCMV DNA was detected in the R051, R155, and R172 monkeys; however, the copy numbers were negligible and Rh CMV DNA was not detected in R173 (Figure 8). In addition, RhCMV reactivation-related symptoms and signs were not observed in any of the recipient monkeys. Substantial anti-RhCMV antibody levels were detected in all recipients throughout the study period and these occasionally increased without significant RhCMV reactivation (Figure 8).

4 | DISCUSSION

Xenotransplantation has been highlighted as an alternative to solve the donor shortage problem of allotransplantation. However, an immunosuppressive regimen that can establish tolerance to xenografts has not been developed. To date, xenotransplantation is used as a bridge for patients who are waiting for allotransplantation. To implement xenotransplantation in the clinic, we need to develop an immunosuppressive regimen that can induce the long-term survival of subsequent allo islets after the rejection of the prior xenotransplantation. In this study, we tested whether a tofacinib-based immunosuppressant regimen provided the long-term survival of allo islets in diabetic NHPs that had already experienced xeno islet rejection.

Indeed, four porcine islet-rejecting diabetic NHP recipients restored normoglycemia either without insulin or with a significantly reduced amount of insulin after intraportal allo islet transplantation under the tofacitinib-based regimen. Serial IVGTTs and immunohistochemical analyses of the liver biopsy samples showed the sustained function of transplanted islets for up to >440 days in one recipient.

Although second infused allo islets showed long-term survival under the tofacitinib-based regimen in three recipients (R144, R172, R174), the first infused allo islets were quickly rejected in R051. We believe that this early failure of the first transplantation in R051 might have resulted from the insufficient control of early inflammation, in this case, IL-1β blockade (anakinra) was not included in the regimen. Because damage to transplanted islets by IL-1β was reported previously,[13] we used anakinra in the other recipients (R144, R172, R173) and improved graft survival was observed in all three recipients (R172, R173, R174).

It seems that R144 has a significant number of IFN-γ producing cells against allo islets during rejection of the xeno islets. Additionally, R144 also showed the worst allogeneic islet function. Although it is difficult to draw conclusions based on one case, allo-sensitization by cross-reacting xeno islet rejection may subsequently compromise the outcome of first allo islet transplantation. R172 and R173, which have low IFN-γ reactivity against alloantigen after xeno islet rejection, showed relatively good glucose control after the first allo islet transplantation compared to R144. Therefore, it appears that reactivity against alloantigen after xeno islet rejection may be the determining factor for the outcome of allo islet transplantation. Furthermore, the

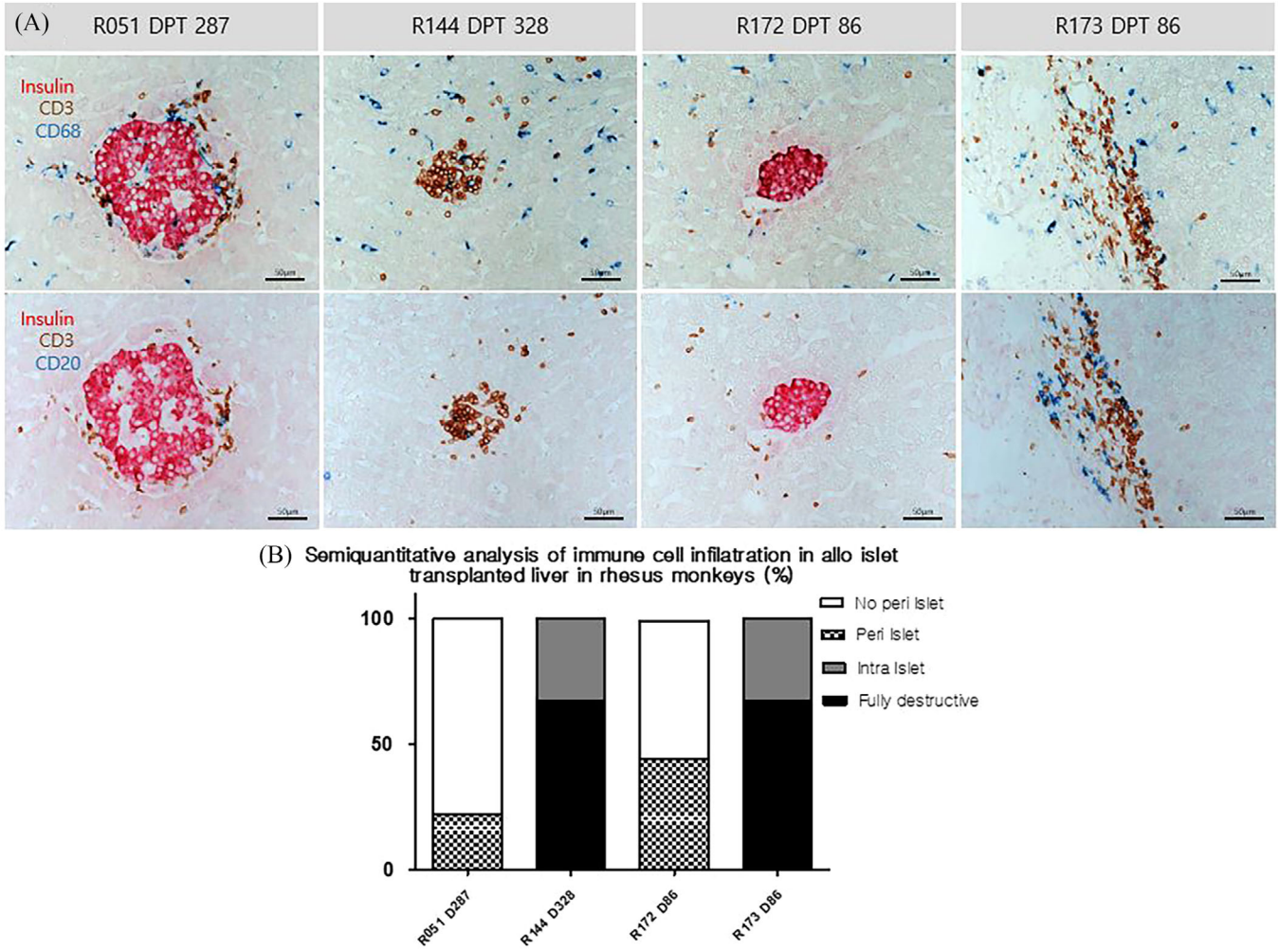

FIGURE 6 Immunohistochemical staining of liver biopsies. The livers were biopsied from R051, R144, R172, and R173 at DPT 287, 328, 86, and 86, respectively, after allo transplantation. Liver samples were analyzed by immunohistochemical staining using anti-insulin, CD3, CD20, and CD68 antibodies with appropriate secondary antibodies to reveal monkey β-cells, T cells, B cells, and macrophages, respectively. (A) Islets from R172 were devoid of immune cell infiltration, including T cells, B cells, and macrophages, whereas some T cells and macrophages were observed around islets in R051. Islets were not observed in R144 and R173 because elective liver biopsies were conducted at unstable blood glucose levels and severe immune cell infiltration was observed. It seems that islets were rejected by these immune cells. (B) The degree of T cell infiltration into the islets was semi-quantitatively assessed using a histopathologic grading system (grade 1: no infiltrates, grade 2: peri-islet infiltrates, grade 3: intra-islet infiltrates, grade 4: fully destructive infiltrates). In cases of well-maintained engrafted allo islets, grades of no peri islet sand peri islets were observed in R051 and R172.

time between porcine islet rejection and allo islet transplantation may be an influencing factor for the outcome of allo islet transplantation, as the time between porcine islet rejection and allo islet transplantation for R172 and R173 were much shorter than those for R051 and R144. The interval time between transplants might be a factor affecting primary graft function. Additionally, crossmatching and donor-specific antibody response also could affect the outcome of transplantation. Because of the small sample size of this study, further research is needed to determine whether time between xeno islet transplant and allo islet transplant can affect primary graft function and how the criteria for allo islet transplantation might affect the successful allograft outcomes.

We compared the results of our previous study,[11] which utilized the same immunosuppression protocol, with the outcomes of the current experiment. It is challenging to make a direct comparison of two studies due to differences in the used animal species (cynomolgus in the previous study vs. rhesus in the current one), and recipients only received the allo islet transplantation alone in previous study, whereas in this experiment, recipients had experienced allo islet transplantation after porcine islet graft rejection. Nevertheless, we found no significant differences in terms of cell number and IFN-γ reactivity between the previous and current study. The most notable distinction lies in the amount of transplanted islet mass. In the previous study, the mean islet mass was about 8600 IEQ/kg, whereas in this experiment, it was about 12 000 IEQ/kg. Consequently, the average graft survival days were higher in this experiment (mean 308 days with four subjects) compared to the previous study (mean 187 days with three subjects). Although the experimental settings between the previous

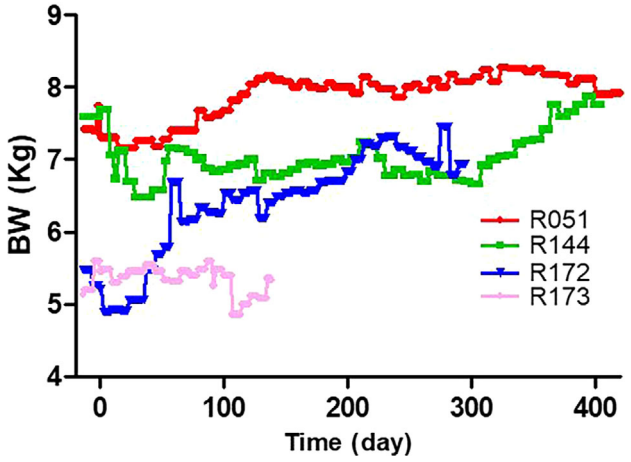

FIGURE 7 Body weights of porcine islet-sensitized diabetic monkeys after allo islet transplantation. All monkeys maintained a normal body weight after allo islet transplantation.

and current experiments were not identical, the consistency in the immunosuppression regimen suggests that the quantity of transplanted islet mass may play a more crucial role in graft survival than cellular reactivity to donor antigens or cross-reactivity. However, due to the limited sample size and differing experimental settings, further research is warranted to confirm these findings. Of note, the tofacitinib-based immunosuppressive regimen also prolonged the survival of the second allo islet transplantation in NHPs that had experienced xeno islet rejection. In general, memory cell responses against donor antigens are more rapid and robust than naïve cell immune responses.[14] Because the tofacitinib-based immunosuppressive regimen also prolonged the survival of the second infused allo islets, we assumed that our regimen could also effectively inhibit the memory responses if transplanted islets were preserved at early phase. Furthermore, our tofacitinib-based immunosuppressive regimen did not induce severe side effects because all recipient monkeys did not show any complications due to the immunosuppression. In this study, we first showed that long-term allo islet survival in diabetic NHPs could be achieved by the tofacitinib-based immunosuppressive regimen after the prior xeno islet rejection. Because we used an immunosuppressive regimen consisting of clinically applicable drugs, we believe that it could be used in clinical trials. A limitation of this study was the lack of a contemporaneous group that had not undergone xeno islet transplantation before allo transplantation, limited mechanistic depth such as absence of humoral response, small group size ($n = 4$), and variability in methods [e.g., survival of first graft (15–1000 days), time between first graft and re-transplantation (11–534 days), number of times (re-transplanted)]. Although we could not compare the survival rates or immunological features of the group that only received allo islets, we examined the features of allo islet transplanted cynomolgus monkeys in a previous study.[11] Because we used a similar tofacitinib-based immunosuppressive regimen in both studies, we could compare these two groups. Consistent with previous studies, $CD3^+$ T cells were the main infiltrating cells in the allo islet rejected site, but we could not confirm whether these infiltrating cells were xeno antigen sensitized cells.

Taken together, we have shown that long-term allo islet survival could be achieved using a tofacitinib-based immunosuppressant regime even after prior xeno islet rejection. These results suggest that the clinical application of porcine islet xenotransplantation is not likely to have a deleterious effect on subsequent allogeneic islet transplantation. We believe that subsequent allo islet transplantation after xeno islet transplantation might be feasible in the future.

AUTHOR CONTRIBUTIONS

J.-M. Kim, S.-J. Kang, S.-H. Hong, J.-S. Shin, B.-H. Min, H.-J. Kim, H.-J. Kang, and J. Ha contributed to data acquisition. J.-M. Kim, S.-J. Kang, and S.-H. Hong contributed to data analysis and interpretation. J.-M. Kim, S.-H. Hong, and C.-G. Park contributed to conception and design. J.-M. Kim, S.-H. Hong, and C.-G. Park contributed to writing and C.-G. Park is responsible for the final approval of the manuscript.

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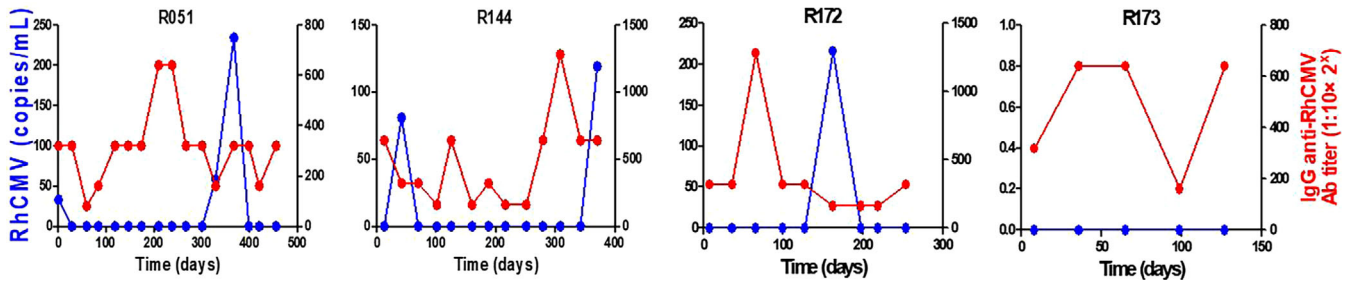

FIGURE 8 Monitoring of RhCMV in the peripheral blood of allo islet transplanted monkeys. The copy number of RhCMV DNA in plasma was determined by quantitative real-time PCR. Anti-RhCMV antibody IgG titers in plasma were assessed by immunofluorescence assay. RhCMV viremias were mild and short in recipients and anti-RhCMV-specific antibodies were consistently detected in the recipients.

CONFLICT OF INTEREST STATEMENT

The authors of this manuscript have no conflicts of interest to disclose as described by the Xenotransplantation.

ORCID

Jong-Min Kim https://orcid.org/0000-0003-0906-0117
Seong-Jun Kang https://orcid.org/0000-0001-8126-4072
So-Hee Hong https://orcid.org/0000-0002-2833-8025
Hyunwoo Chung https://orcid.org/0000-0001-9103-0560
Jun-Seop Shin https://orcid.org/0000-0001-5142-2818
Byoung-Hoon Min https://orcid.org/0000-0003-1597-9517
Hyun Je Kim https://orcid.org/0000-0002-4184-6702
Jongwon Ha https://orcid.org/0000-0003-2285-3517
Chung-Gyu Park https://orcid.org/0000-0003-4083-8791

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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