의학박사 학위논문

The efficacy of transgenic miniature pig corneas and clinically applicable predictive biomarkers for graft rejection in pig-to-nonhuman primates full-thickness corneal xenotransplantation

돼지-영장류 전층 이종각막이식에서 형질전환 미니돼지 각막의 유효성과 임상적용 가능한 거부반응 예측 바이오마커에 관한 연구

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The efficacy of transgenic miniature pig corneas and clinically applicable predictive biomarkers for graft rejection in pig-to-nonhuman primates full-thickness corneal xenotransplantation

by

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ABSTRACT

Purpose: Corneal xenotransplantation using pig donors has been investigated as a substitute for human donor corneas. In this study, we investigated long-term survival of corneal grafts from α1,3-galactosyltransferase gene-knockout miniature (GTKOm) pigs in nonhuman primates (NHPs). We also investigated the clinically applicable predictive biomarkers for corneal xenograft rejection including the results of our previous experiments.

Methods: For GTKOm survival study, nine rhesus macaques undergoing full-thickness corneal xenotransplantation using GTKOm pigs were systemically administered steroid, basiliximab, intravenous immunoglobulin, and tacrolimus with (CD20 group; n = 4) or without (control group; n = 5) anti-CD20 antibody. The graft score (0-12) was calculated based on opacity, edema, and vascularization. Scores ≥ 6 were defined as rejection. Changes in T/B cell subsets, levels of anti-αGal IgG/M, donor-specific IgG/M from blood, and C3a from plasma and aqueous humor (AH) were evaluated. For biomarker study, 34 NHPs which had undergone full-thickness porcine corneal xenotransplantation were included. Five of them received GTKOm pig corneas, and 29 received SNU wild type miniature pig corneas. They were divided into two groups: (a) graft rejection within 6 months (rejection group); and (b) graft survival until 6 months (survival group). The entire rejection group included all NHPs whose graft was rejected within a 6-month period, while late rejection group included NHPs whose graft was rejected after

more than 4 weeks up to 6 months. None of the NHPs showed rejection at postoperative week 2. In the evaluation of the 2-week biomarkers, entire rejection group (n = 16) or late rejection group (n = 12) was compared to survival group (n = 18). In the evaluation of 4-week biomarkers, four NHPs showing rejection within 4 weeks were excluded and late rejection group (n = 12) was compared to survival group (n = 18). Analysis of biomarker candidates included T/B cell subsets, levels of anti-αGal IgG/M, donor-specific IgG/M from blood, and C3a from plasma and aqueous humor (AH).

Results: In GTKOm survival study, graft survival was significantly longer (P = 0.008) in the CD20 group (>375, >187, >187, >83 days) than control group (165, 91, 72, 55, 37 days). Activated B cells were lower in the CD20 group than control group (P = 0.026). Aqueous humor complement C3a was increased in the control group at last examination (P = 0.043), and was higher than that in the CD20 group (P = 0.014). At last examination, anti-non-Gal IgG was increased in the control group alone (P = 0.013). In biomarker study, $CD8^{+}IFN\gamma^{+}$ cells at week 2 and AH C3a at week 4 were significantly elevated in the rejection group. Receiver operating characteristic areas under the curve was highest for AH C3a (0.847) followed by $CD8^{+}IFN\gamma^{+}$ cells (both the concentration and percentage: 0.715), indicating excellent or acceptable discrimination ability

Conclusion: The GTKOm pig corneal graft achieved long-term survival when combined with anti-CD20 antibody treatment. Inhibition of activated B cells and

complement is imperative even when using GTKO pig corneas. $CD8^{+}IFN\gamma^{+}$ cells at week 2 and AH C3a at week 4 are reliable biomarkers for predicting rejection in pig-to-NHP corneal xenotransplantation. Those biomarkers may be used as a standard of reference to predict rejection in clinical trials of corneal xenotransplantation.

Keywords: anti-CD20 antibody, biomarker, rejection, cornea, xenotransplantation, α1,3-galactosyltransferase gene knockout miniature pig, nonhuman primate

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LIST OF ABBREVIATIONS

Ab, antibody

αGal, Galalpha1,3Galbeta1,4GlcNAc-R

AH, aqueous humor

AU, artificial unit

AUC, areas under the curve

C3a, complement activation fragment

CCT, Central corneal thickness

DS, donor pig-specific

ECD, Endothelial cell density

GT, α1,3-galactosyltransferase

GTKO, α1,3-galactosyltransferase gene knockout

GTKOm, α1,3-galactosyltransferase gene knockout miniature

H&E, Hematoxylin and eosin

IFN, interferon

IL, interleukin

IOP, Intraocular pressure

IVIG, intravenous immunoglobulin

Kim, Knock-in miniature

MFI, mean fluorescence intensity

NHP, non-human primate

POD, Postoperative day

ROC, receiver operating characteristic

SE, Standard error

SNU, Seoul National University

WT, wild type

CHAPTER 1

Long-term survival of full thickness corneal xenografts from α1,3-galactosyltransferase gene-knockout miniature pigs in nonhuman primates

INTRODUCTION

Corneal blindness causes global vision loss, and corneal transplantation is the treatment of choice.[1-3] Unfortunately, the demands for transplantation exceed donor availability in developing countries. Therefore, corneal xenotransplantation has been studied as an alternative treatment.[4] The Galα1,3Galβ1,4GlcNAc-R(αGal) epitope is a major xenoantigen in solid organ xenotransplantation synthesized by α1,3-galactosyltransferase (GT).[5,6] Xenograft survival is prolonged with α1,3-galactosyltransferase gene knockout (GTKO) pig organs.[5,6] Although the cornea expresses less αGal than that expressed by other organs, evidence suggests that αGal is involved in xenogeneic rejection of corneal grafts.[7,8]

By eliminating αGal-related reaction, GTKO pig corneal grafts should survive longer than wild type (WT) grafts.[9] However, previous studies have reported otherwise in non-human primates (NHPs).[10-12] Although the rejection mechanism in GTKO pig corneal xenotransplantation remains controversial, there are two possibilities for graft failure: (1) antibody-mediated rejection may still occur although GTKO lacks αGal or (2) disparity of the corneal thickness may affect survival. Donor and recipient corneal thickness must be matched for optimal wound approximation. Genetically engineered non-miniature pig cornea is thicker than miniature pig cornea of the same age, therefore younger donors (≤ 3-month-old) were used in previous studies.[10,11] However, younger donor corneas are too flaccid to handle properly, resulting in severe inflammation.[10] Miniature pigs allow older donors (> 7-month-old) to be used with appropriate thickness match.

In this study, we conducted full-thickness corneal xenotransplantation in NHPs using GTKO miniature (GTKOm) pigs with a mean age of 11.8 months (1.5-24-month-old) with or without anti-CD20 antibody (Ab) treatment to evaluate whether GTKOm pig can prolong graft survival. Regardless of donor age, long-term survival of the graft depended on the administration of anti-CD20 Ab treatment. To our knowledge, this is the first report demonstrating graft survival beyond 6 months in GTKOm pig-to-NHP full thickness corneal xenotransplantation.

MATERIALS AND METHODS

This study adhered to ARVO Statement regarding the Use of Animals in Ophthalmic and Vision Research. Study protocol was approved by Research Ethics Committee at Seoul National University Hospital (IACUC No. 15-0171).

We used 14 eyes from seven GTKOm pigs: four GTKOm (three from Optipharm Inc. and one from National Institute of Animal Science, South Korea); two GTKO/hCD39 knock-in miniature (KIm; from Optipharm Inc.); and one GTKO/CMAH/iGb3s triple knock-out miniature (TKOm; from Optipharm Inc.) pigs.[13,14] Among them, nine eyes were used for *in vivo* transplantation to evaluate efficacy and the remaining five corneas (four from GTKOm and one from GTKO/CD39 KIm) were used for *in vitro* studies to evaluate bio-physical characteristics, such as endothelial density changes over time, endothelial cell proliferation, and the presence of ATPase pump by immunofluorescence staining; these results are presented in Figure 1.1. Before allocating corneas into the *in vivo* or *in vitro* studies, we measured the optical properties of six of the eyes (Table 1.1).

Nine Chinese rhesus macaques were divided into two groups based on the administration of anti-CD20 Ab. In the control group, five NHPs (four with GTKOm and one with GTKO/hCD39 KIm corneas) were systemically administered steroid, basiliximab, intravenous immunoglobulin (IVIG), and tacrolimus. In the CD20 group, four NHPs (two GTKOm and two TKOm corneas) were systemically administered steroid, basiliximab, IVIG, tacrolimus, and anti-CD20 Ab.

Pig-to-NHP full-thickness corneal transplantation

All corneal grafts sized of 7.5 mm were transplanted in each right eye of the rhesus macaque, trephined with 7.0 mm using the same keratoplasty technique described previously.[15,16] Immediately after the surgery, dexamethasone 1.5 mg/0.3mL (JW Pharmaceutical, Seoul, Republic of Korea) and 0.25 mL of aflibercept (EYLEA®, Regeneron Pharmaceuticals, Inc., Tarrytown, New York) were injected subconjunctivally.

Postoperative topical and systemic immunosuppressive regimen

Postoperatively, all nine NHPs received the following medications. Levofloxacin 0.5% (Cravit®, Santen Pharmaceutical, Osaka, Japan) and prednisolone acetate 1% (Pred forte®, Allergan, Irvine, CA) were topically administered once daily. Dexamethasone 1.5 mg/0.3 mL (JW Pharmaceutical) was injected weekly subconjunctivally. Methylprednisolone (Solu-medrol®, Pfizer, New York, NY) was injected intramuscularly at an initial dose of 2 mg/kg/day. It was tapered over five weeks and discontinued at a final dose of 0.25 mg/kg. Basiliximab (0.3 mg/kg; Simulect®, Novartis Pharmaceuticals Corporation, East Hanover, NJ) was intravenously administered on days 0 and 4 after transplantation. IVIG (1 g/kg) was administered on day 1 and at 2 weeks after transplantation. Tacrolimus (Prograf®, Astellas Pharma US, Deerfield, IL) was intramuscularly injected twice daily with a dose of 0.035 mg/kg (control) or 0.05 (CD20 group) from two days before and up to 6 months after the surgery. The dosage of the tacrolimus was

temporarily adjusted (tapering or intermittent discontinuation) accordingly when the systemic deterioration was detected in the recipients.

Anti-CD20 Ab (MabThera®, Hoffmann-La Roche, Basel, Switzerland) was administered twice in the first week (D0 and D7) and then every two months only in CD20 group, which is the same protocol in our previous report.[16]

Postoperative intra-group and inter-group analysis for a survival and immunologic profile changes

Clinically, graft survival and increased intraocular pressure (IOP) were monitored. Graft score (0-12) was calculated as the sum of grades for opacity, edema, and vascularization measured by slit-lamp microscopy.[17] Graft scores ≥ 6 were considered as rejection. IOP and central corneal thickness (CCT) were measured using a Tono-Pen (Medtronic Solan, Jacksonville, FL) and an ultrasonic pachymeter (Quantel Medical, Clermont-Ferrand, France), respectively. Recipients diagnosed with graft rejection were sacrificed within two weeks. The recipients with survived grafts were monitored up to 6 months. Graft survival was compared based on the administration of anti-CD20 Ab (control vs CD20 group) or donor pig age (≤ 7-month-old vs > 7-month-old). The fluctuation of lymphocyte, complement, and antibody levels were compared pre- and post-operatively within each group and between the CD20 and control groups at three time points: preoperatively, four weeks postoperatively, and at the last examination. Last examination was defined as the time of sacrifice in rejected recipients or the final follow-up in survived recipients.

Flow cytometry-based T and B cell assay

T cell sub-populations (interferon-γ^+[IFNγ^+], $CD28^+CD95^+$ central memory, $CD28^-CD95^+$ effector memory, and $CD4^+CD25^+Foxp3^+$ regulatory cells) and B cell populations ($CD3^-CD20^+$ B and $CD3^-CD20^+CD28^+$ activated B cells) were evaluated using flow cytometry analysis of whole blood as previously mentioned.[15,18] For extracellular surface staining, cell suspensions were incubated at 4°C for 30 minutes with fluorescein-conjugated mouse anti-human Abs as follows: CD3-FITC (1:40), CD4-FITC (1:200), CD8-PerCp-Cy5.5 (1:200), CD25-APC, CD28-APC (1:200), CD95-PE (1:200), and CD20-PE (1:200). For intracellular Ab staining, cell suspensions were incubated at 4°C with fluorescein-conjugated mouse anti-human Abs as follows: IFN-γ-PE (1:200, 30 minutes) and Foxp3-PE (1:200, one hour). Intracellular IFN-γ staining was performed after stimulation overnight with anti-CD28 Ab (0.25 μg/ml) and anti-CD3 Ab (2.5 μg/ml) in the presence of GolgiPlug (brefeldin A; 1 μl/1ml). All Abs were purchased from eBioscience (San Diego, CA, USA), except CD3-FITC (BD PharMingen, San Diego, CA, USA) and anti-CD3 Ab (U-CyTech, Utrecht, The Netherlands). Data were acquired using a FACSCanto flow cytometer (Becton-Dickinson, Mountain View, CA, USA) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Complement (C3a) and antibody assay

Concentrations of C3a in the plasma and aqueous humor (AH) were measured

using commercial ELISA kits (BD OptEIA™ Human C3a ELISA Kit; BD Biosciences, San Diego, CA) according to the manufacturer's protocols.

Plasma anti-αGal IgG/IgM Abs were measured by ELISA as previously described.[19,20] To determine anti-non-Gal IgG Ab-binding responses, flow cytometry was used with a GTKO porcine endothelial cell line (PEC69). For supplemental purposes, WT porcine endothelial cells (MPN-3) were incubated with 1/10 diluted plasma samples. The level of Ab binding was determined as net mean fluorescence intensity (nMFI) calculated by subtracting MFI value of porcine plasma (negative control) from MFI value of the sample. Plasma levels of donor pig-specific IgM/IgG Abs were measured by flow cross-match technique as described previously[18] using donor peripheral blood mononuclear cell as targeting cells.

Immunofluorescence staining

Corneas from sacrificed recipients were subjected to hematoxylin and eosin (H&E) staining and immunofluorescent staining. Stainings for $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD3^-CD20^+$ B cells, $CD68^+$ macrophages, C3c, and IgG were carried out as previously described.[7,16,18] The primary Abs used are as follows: anti-CD3 Ab (1:200; Abcam, Cambridge, MA), anti-CD4-alexa Fluor 488 conjugated Ab (1:50; Novusbio, Littleton, CO), anti-CD8 Ab (1:150; Abcam), anti-CD20 Ab (1:100; eBioscience, San Diego, CA), anti-CD68 Ab (1:100; Thermo Scientific, Runcorn, United Kingdom), anti-C3c Ab (A0062; 1:100, DAKO, Glostrup, Denmark), and anti-IgG (Fc specific) Ab (AP31438FC-N; 1:20, Acris Antibodies,

Inc., San Diego, CA).

Statistical analysis

Kaplan–Meier survival test was used for graft survival analysis, Friedman test for intra-group time dependent analysis, and Mann-Whitney U test for inter-group analysis. Statistical significance was accepted at $P< 0.05$. Data are presented as mean ± standard error (SE). GraphPad Software (GraphPad Prism, Inc., La Jolla, CA) was used for statistical analyses.

Table 1.1. Physical and optical properties of cornea in GTKOm pigs (N = 6)

	GTKOm pig	SNU WT miniature pig[30]	Human[30]	P*
Age (months)	11.25 ± 3.37	41.73 ± 3.62 mm	NA	<0.001
Horizontal corneal diameter	18.0 ± 0.0 mm	17.1 ± 0.4 mm	11.4 ± 0.7 mm	<0.001
Vertical corneal diameter	16.5 ± 0.9 mm	15.5 ± 0.5 mm	10.5 ± 0.5 mm	0.001
Axial length (IOLMaster)	19.33 ± 2.45 mm	23.12 ± 0.97 mm	23.58 ± 1.13 mm	<0.001
Sim K maximum value (Orbscan)	44.5 ± 6.2 D	37.4 ± 1.8 D	44.0 ± 2.4 D	0.002
Sim K minimum value (Orbscan)	41.8 ± 5.9 D	35.2 ± 1.6 D	42.1 ± 1.8 D	0.001
Sim K astigmatism (Orbscan)	2.6 ± 0.7 D	2.3 ± 0.9 D	1.1 ± 0.9 D	0.986
ACD (AS-OCT)	2.39 ± 0.57 mm	3.18 ± 0.41 mm	3.64 ± 0.33 mm	0.021
CCT (ultrasound pachymetry)	762.9 ± 137.4 ㎛	852.6 ± 65.2 ㎛	542.3 ± 36.7 ㎛	0.099

* Between GTKOm vs. SNU miniature pigs, Mann-Whitney test

Data are presented as mean ± standard deviation. GTKOm, α1,3-galactosyltransferase gene knockout miniature; SNU, Seoul National University; WT, wild type; K, keratometric value; Sim K, simulated keratometric value in corneal topography; D, diopter; ACD, anterior chamber depth; AS-OCT, anterior segment optical coherence tomography; CCT, central corneal thickness.

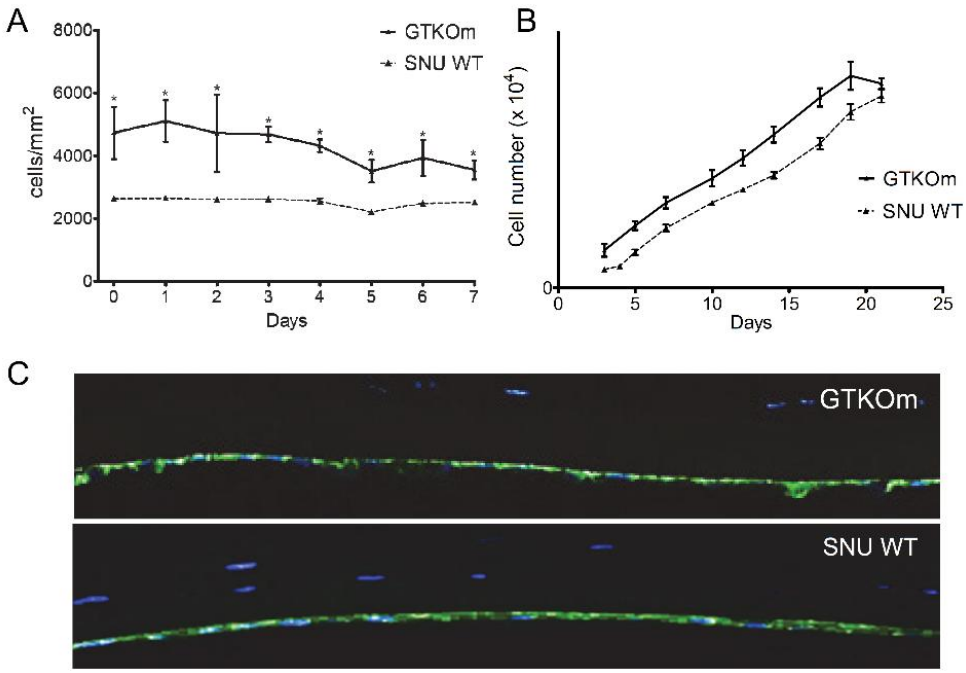


Figure 1.1. (A) Serial changes of corneal endothelial cell density (ECD) of GTKOm pigs (n = 5, mean age = 7 months) and SNU WT miniature pigs (adopted from previous study[21]); n = 23, mean age = 37 months) corneas after preservation in Optisol GS. Mean ECD of GTKOm pigs was higher than that of SNU miniature pigs (all $P< 0.05$, Mann-Whitney test). (B) Proliferative capacity of corneal endothelial cells in GTKOm and SNU WT miniature pigs. Proliferative capacities of endothelial cells of GTKOm were comparable to those of SNU WT miniature pigs ($P = 0.403$, Mann-Whitney test). (C) Immunofluorescence staining of Na- and K-dependent ATPase pump in GTKOm and SNU WT miniature pig cornea. Na- and K-dependent ATPase pumps in both groups were well observed. All data are described as mean ± standard error. GTKOm, α1,3-galactosyltransferase gene knockout miniature; SNU, Seoul National University; WT, wild type; *$P< 0.05$.

RESULTS

GTKOm corneal grafts survived longer with anti-CD20 Ab treatment

Table 1.2 shows the demographic data of the recipients and donors. The CD20 group showed significantly longer graft survival (>375, >187, >187, >83 days) than the control group (165, 91, 72, 55, 37 days; $P = 0.008$, log-rank test; Figures 1.2 and 1.3A). One recipient in the CD20 group (No. 2.) died on postoperative day (POD) 83 secondary to immunosuppression-related microangiopathy, at which point the graft was transparent. When we compared the graft survival time between older (> 7-month-old) and younger (≤ 7-month-old) aged donor recipients, there was no significant difference (Figure 1.3B).

The CCT was well-maintained in the CD20 group, while two recipients in the control group showed increased thickness associated with graft rejection (Figure 1.3C). Transient IOP elevation occurred in one recipient, but was managed with anti-glaucoma treatment (Figure 1.3D). Within the first postoperative month, six recipients (three from each group) developed thin retrocorneal membranes, which resolved within 12-50 days (28.5 ± 6.9 days), restoring corneal transparency. Thick retrocorneal membranes that developed around the rejection period were permanent in two recipients in the control group (No. 2 and No. 5). The incidence of those retrocorneal membranes (thin or thick) was not associated with donor pig age ($P = 0.262$ or 0.333, respectively). Notably, peripheral stromal melting progressed near the recipient site of the graft junction in four recipients with rejected grafts in the control group (Figure 1.4).

After six months, surviving grafts showed well-maintained endothelial

cell density (Figure 1.5A). Anterior segment optical coherence tomography and corneal topography examinations showed well-adapted graft-recipient junctions, open angles and deep anterior chambers, and acceptable optical properties without edema (Figures 1.5B-D).

Reduction of B cells in GTKOm pig-to-NHP corneal xenotransplantation treated with anti-CD20

The levels of IFNγ^+, effector memory and central memory $CD4^+/CD8^+$ T cells, and regulatory T cells remained unchanged before and after surgery in each group (intra-group analysis) and were not significantly different between the two groups at any of the time points (inter-group analysis, Figure 1.6). The level of B cells was significantly lower in the CD20 group than in the control group at the 4-week follow-up and last examination (all $P = 0.014$; Figure 1.7A). The level of activated B cells was also significantly lower in the CD20 group than in the control group at the last examination ($P = 0.026$). Compared to baseline, levels of activated B cells were significantly lower at 4 weeks in the control group ($P = 0.034$) and at the last examination in the CD20 group ($P = 0.014$, Figure 1.7B).

Increased complement (C3a) and anti-non-Gal IgG were observed in GTKOm pig-to-NHP corneal xenotransplantation without anti-CD20 Ab treatment

The level of C3a in the AH was significantly higher in control group than in the CD20 group at the last examination ($P = 0.029$, Figure 1.7D). Complement levels in the control group were significantly higher at the last examination than at the

baseline ($P = 0.008$), while levels were unchanged in CD20 group across all time points.

Although donor-specific IgM levels were significantly higher in the CD20 group than in the control group preoperatively, they were stable during follow-up examination in either group (Figure 1.8A). Levels of anti-αGal IgM and IgG were not increased in either of the groups (Figures 1.8B and 1.8E). Also of note, levels of anti-GTKO PEC IgG (anti-non-Gal IgG) were significantly increased from baseline in the control group at the last examination ($P = 0.013$, Figure 1.8C), while they were unchanged in the CD20 group.

Histology of GTKOm corneal grafts

αGal was not expressed in GTKOm cornea. Human CD39 was well-expressed in hCD39 KI cornea (Figure 1.9).

Rejected grafts showed diffuse inflammatory cell infiltration at the graft junction and thick retrocorneal membrane formation (Figures 1.10A and 1.10B) while the graft junction in surviving grafts had much less infiltrating inflammatory cells (Figure 1.10C).

Immunofluorescence staining presented extensive infiltration of $CD3^+CD4^+$ T, $CD3^+CD8^+$ T, $CD3^-CD20^+$ B lymphocytes and $CD68^+$ macrophages at the graft junction in rejected grafts (Figure 1.11A) as well as in the retrocorneal membranes (Figure 1.11B), while few inflammatory cells were identified in surviving grafts (Figure 1.11C). Compared with a surviving grafts, rejected grafts showed dense depositions of C3c and IgG at the graft junction and in the

retrocorneal membranes (Figure 1.11).

Table 1.2. Demographic data of the recipients and donors, systemic immunosuppressive regimen, and graft survival.

Recipient rhesus			Donor pig					Graft survival (days)
Name	Age (mo)	Systemic Immunosuppression	Genotype	Strain	Age (mo)	CCT (μm)	ECD (number/mm^3)	
Control No. 1	55	Steroid + Basiliximab + IVIG + Tacrolimus[a]	GTKOm	White Yucatan	24	914	2604	55
Control No. 2	52				15	831	2874	37
Control No. 3	52					806	2865	72
Control No. 4	54			Crossbreeding Landrace with Chicago minipig	4.5	595	4184	165
Control No. 5	56		GTKO/ hCD39KIm	White Yucatan	1.5	707	6135	91
CD20 No. 1	59	Steroid + Basiliximab + IVIG + Tacrolimus[b] + Anti-CD20 Ab	GTKOm	White Yucatan	19	898	2381	>365
CD20 No. 2	59					919	2381	>83
CD20 No. 3	59		GT/CMAH/i Gb3s TKOm	White Yucatan	7	767	3546	>187
CD20 No. 4	58					752	3472	>187

Methylprednisolone (Solu-medrol®, Pfizer, New York, NY) was injected intramuscularly at an initial dose of 2 mg/kg/day. It was tapered over five weeks and discontinued at a final dose of 0.25 mg/kg.

Basiliximab (0.3 mg/kg; Simulect®, Novartis Pharmaceuticals Corporation, East Hanover, NJ) was intravenously administered on days 0 and 4 after transplantation.

IVIG (1 g/kg) was administered on day 1 and at 2 weeks after transplantation.

Tacrolimus (Prograf®, Astellas Pharma US, Deerfield, IL) was intramuscularly injected twice daily with a dose of 0.035 mg/kg (control group)[a] or 0.05 (CD20 group)[b]from two days before and up to 6 months after the surgery.

Anti-CD20 Ab (MabThera®, Hoffmann-La Roche, Basel, Switzerland) was administered twice in the first week (D0 and D7) and then every two months only

in CD20 group.

IVIG, intravenous immunoglobulin, mo, month; CCT, central corneal thickness; ECD, endothelial cell density; GTKOm, α1,3-galactosyltransferase gene knockout miniature; hCD39KIm, human CD 39 knock-in miniature; TKO, triple knock-out miniature

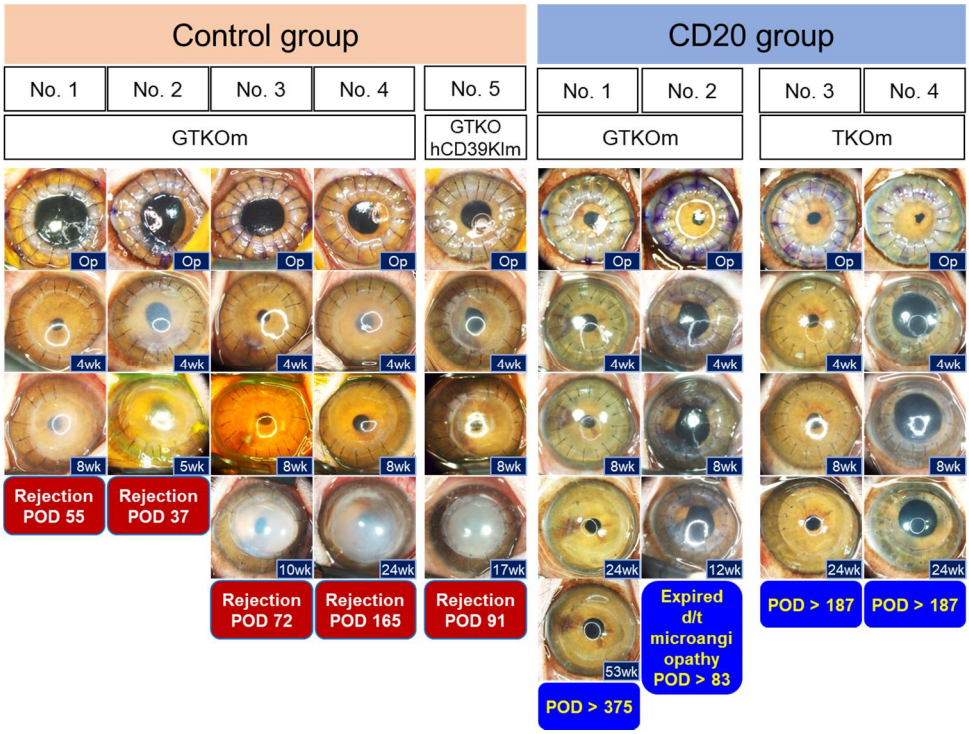


Figure 1.2. Representative photographs of corneal xenografts in NHPs. The CD20 group showed significantly longer graft survival (>365, >187, >187, >83 days) than the control group (165, 91, 72, 55, 37 days). One recipient in the CD20 group died on postoperative day 83 secondary to immunosuppression-related microangiopathy, at which point the graft was transparent. GTKOm, α1,3-galactosyltransferase gene-knockout miniature; hCD39KIm, human CD 39 knock-in miniature; TKOm, GTKO/CMAH/iGb3s triple knock-out miniature; IVIG, intravenous immunoglobulin.

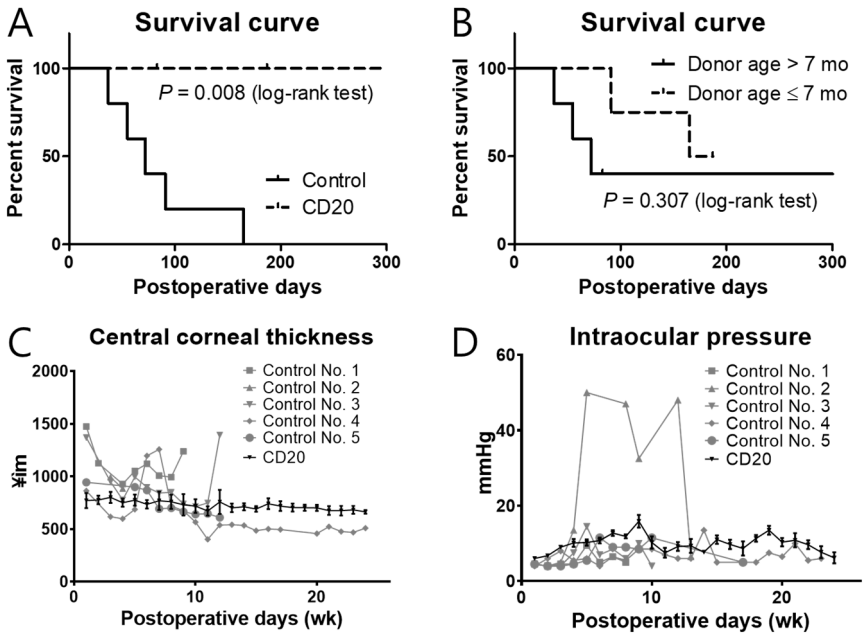


Figure 1.3. Graft survival curves according to treatment with anti-CD20 Ab (A) or donor pig age (≤ 7-month-old vs > 7-month-old) (B). (A) There was a significantly prolonged survival time in the anti-CD20 group compared with that in the control (P = 0.008, log- rank test). The black solid line indicates control and dotted line indicates CD20 groups. (B) There was no significant difference in survival based on donor age. The black solid line indicates older donor pigs (more than 7 months of age) and dotted line indicates young donor pigs (equal to or less than 7 months of age). (C) The central corneal thickness increased after rejection in two recipients in the control group (No. 1 and 3). The central corneal thickness was well maintained in the CD20 group during the follow-up (black line). (D) Intraocular pressure was well maintained within the normal range in most recipients; transient elevation occurred in one recipient, but was managed with anti-glaucoma treatment (control No. 2). All data were described as mean ± standard error.

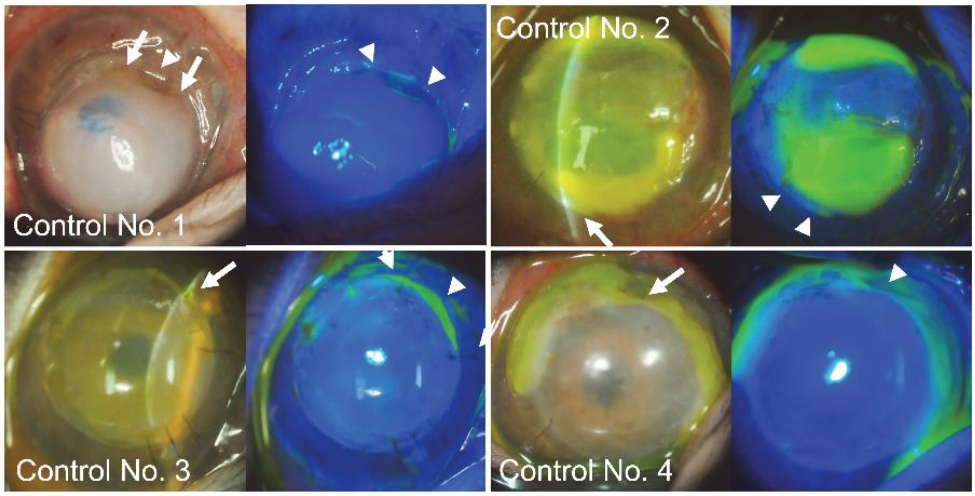


Figure 1.4. Representative white and fluorescein photographs of corneal melting on the recipient side of the junction in rejected grafts using slit beam microscopy. Around graft-recipient junction (white arrow), recipient stromal melting (white arrowhead) was observed near the rejection period.

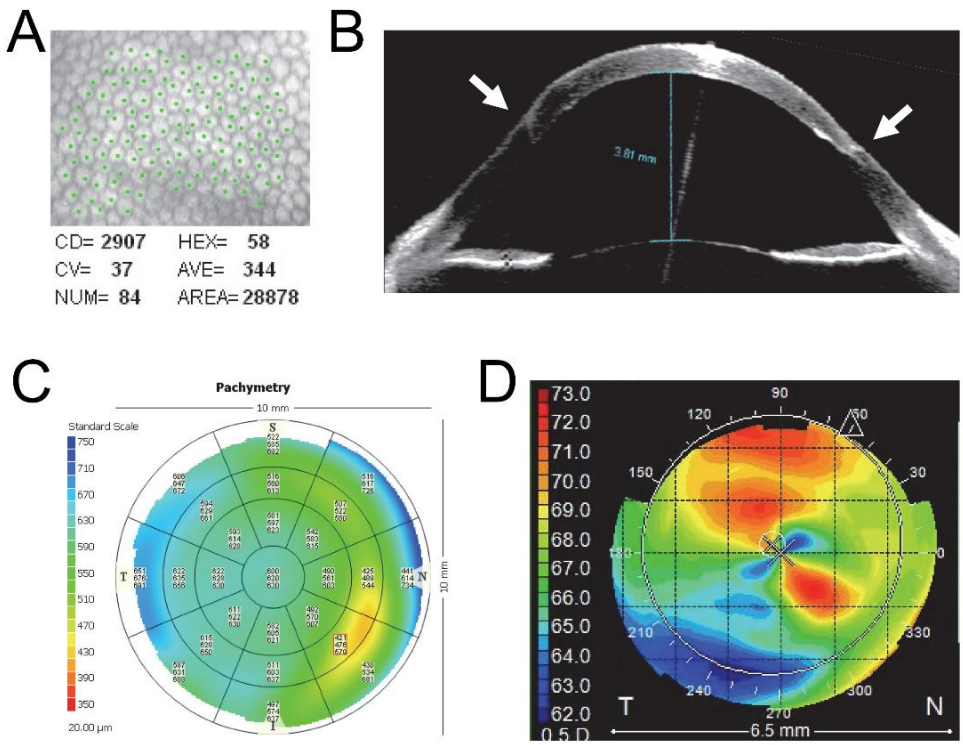


Figure 1.5. Representative photographs of a surviving graft immediately after sacrifice (postoperative 187 days) demonstrating acceptable optical properties. (A) Specular microscopy shows endothelial cell density was well-maintained over 2900/mm^2. (B) Cross-sectional image in AS-OCT shows well-approximated thickness-matched graft-recipient junction (white arrows), well-preserved depth of anterior chamber and open angle. (C) Pachymetry map in AS-OCT shows graft thickness was within normal ranges. (D) Topographic image shows an astigmatic graft. AS-OCT, anterior segment optical coherence tomography.

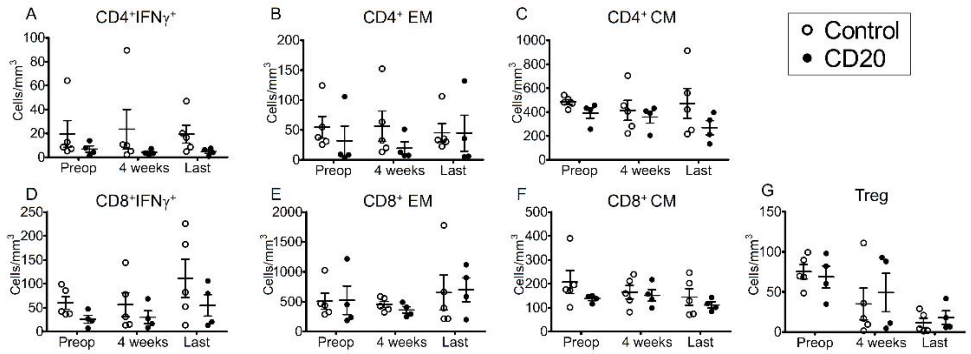


Figure 1.6. Comparative analysis of T cell responses between the control and CD20 groups. The levels of IFNγ^+, effector and central memory $CD4^+$/$CD8^+$ T cells, and regulatory T cells remained unchanged before and after surgery in each group and were not significantly different between the two groups at any of the time points. All data are described as mean ± standard error. EM, effector memory; CM, central memory; Treg, regulatory T cell. "Preop" indicates pre-operative baseline data. "Last" indicates data at the last examination, and $*P < 0.05$. The last examination was defined as the time of sacrifice in rejected recipients or the last follow-up in survived recipients.

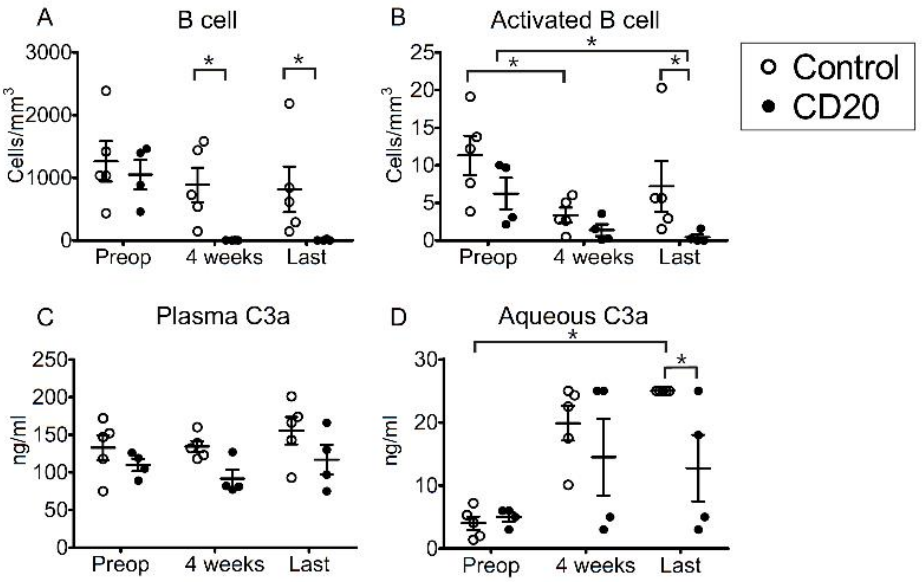


Figure 1.7. Comparative analysis of B cells and complement (C3a) between the control and CD20 groups. (A) The level of B cells was significantly lower in the CD20 group than in the control group at the 4-week follow-up and last examination (all $P = 0.014$, Mann-Whitney test). (B) The level of activated B cells was also significantly lower in the CD20 group than in the control group at the last examination ($P = 0.026$, Mann-Whitney test). Compared to baseline, levels of activated B cells were significantly lower at 4 weeks in the control group ($P = 0.034$, Friedman test and Dunn multiple comparison test) and at the last examination in the CD20 group ($P = 0.014$, Friedman test and Dunn multiple comparison test). (C) The levels of plasma C3a were not different between the groups and were not changed between pre-operation and 4 weeks/last examination in each group. (D) The level of C3a in the aqueous humor was significantly higher in control group than in the CD20 group at the last examination ($P = 0.029$, Mann-

Whitney test). Complement levels in the control group were significantly higher at the last examination than at the baseline ($P = 0.008$, Friedman test and Dunn multiple comparison test), while levels were unchanged in CD20 group across all time points. All data are described as mean ± standard error. “Preop” indicates pre-operative baseline data. “Last” indicates data at the last examination, and $*P < 0.05$. The last examination was defined as the time of sacrifice in rejected recipients or the last follow-up in survived recipients.

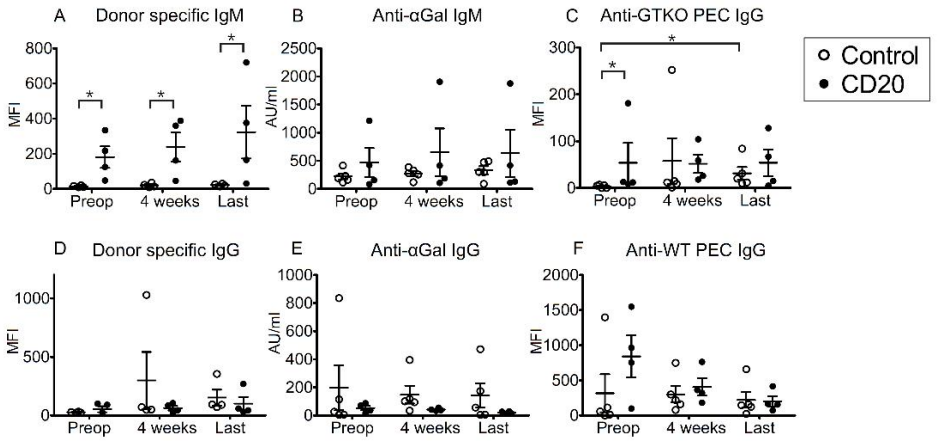


Figure 1.8. Comparative analysis of donor pig-specific antibody (A, D), anti-αGal antibody (B, E), and IgG against GTKO PEC (C) or against wild-type PEC (F) between the control and CD20 groups. (A, D) Although donor-specific IgM levels were significantly higher in the CD20 group than in the control group preoperatively ($P = 0.021$, Mann-Whitney test), they were stable during follow-up examination in either group. (B, E) Levels of anti-αGal IgM and IgG were not increased in either of the groups. (C, F) Levels of anti-GTKO PEC IgG (anti-non-Gal IgG) were significantly increased from baseline in the control group at the last examination ($P= 0.013$, Friedman test and Dunn multiple comparison test), while they were unchanged in the CD20 group. There were no significant changes in MFI values against wild-type PEC in both groups (F). All data are described as mean ± standard error. GTKO, α1,3-galactosyltransferase gene knockout; PEC, porcine endothelial cell; WT, wild-type. "Preop" indicates pre-operative baseline data. "Last" indicates data at the last examination, and $*P < 0.05$. The last examination was defined as the time of sacrifice in rejected recipients or the last follow-up in survived recipients.

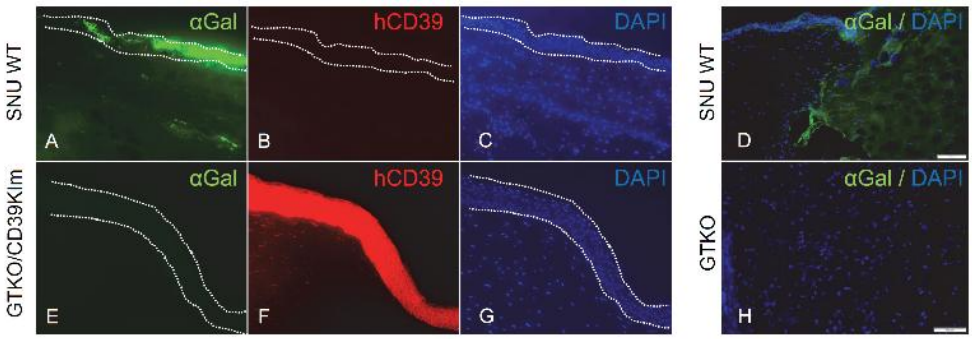


Figure 1.9. Corneal immunofluorescence staining of αGal (A, E) and human CD39 (B, F) expression in SNU WT (upper) and GTKO/human CD39 knock-in miniature (CD39KIm, lower) pigs, respectively. Dash lines indicate corneal epithelial layer (epithelial side up). SNU WT cornea expressed αGal without expressing human CD39 while GTKO/CD39KI cornea well expressed hCD39 without expressing αGal. (D, H) αGal staining at recipient (left) – graft (right) junction. SNU WT pig graft showed positive staining for αGal. However, recipient (Rhesus) or GTKO pig cornea did not show positive staining for αGal (magnification × 200). For αGal epitopes, the Griffonia simplifolia I isolectin B4 (GSIB4; Molecular Probes, Eugene, OR) conjugated with Alexa Fluor 488 (I-21411; Molecular Probes) was used. For human CD39, anti-human CD39 Ab (mouse monoclonal IgG, Santa Cruz biotechnology, Dallas, TX, USA) was used. SNU, Seoul National University; WT, wild type; GTKO, α1,3-galactosyltransferase gene knockout; αGal , Galα1,3Galβ1,4GlcNAc-R; hCD39KIm, human CD 39 knock-in miniature.

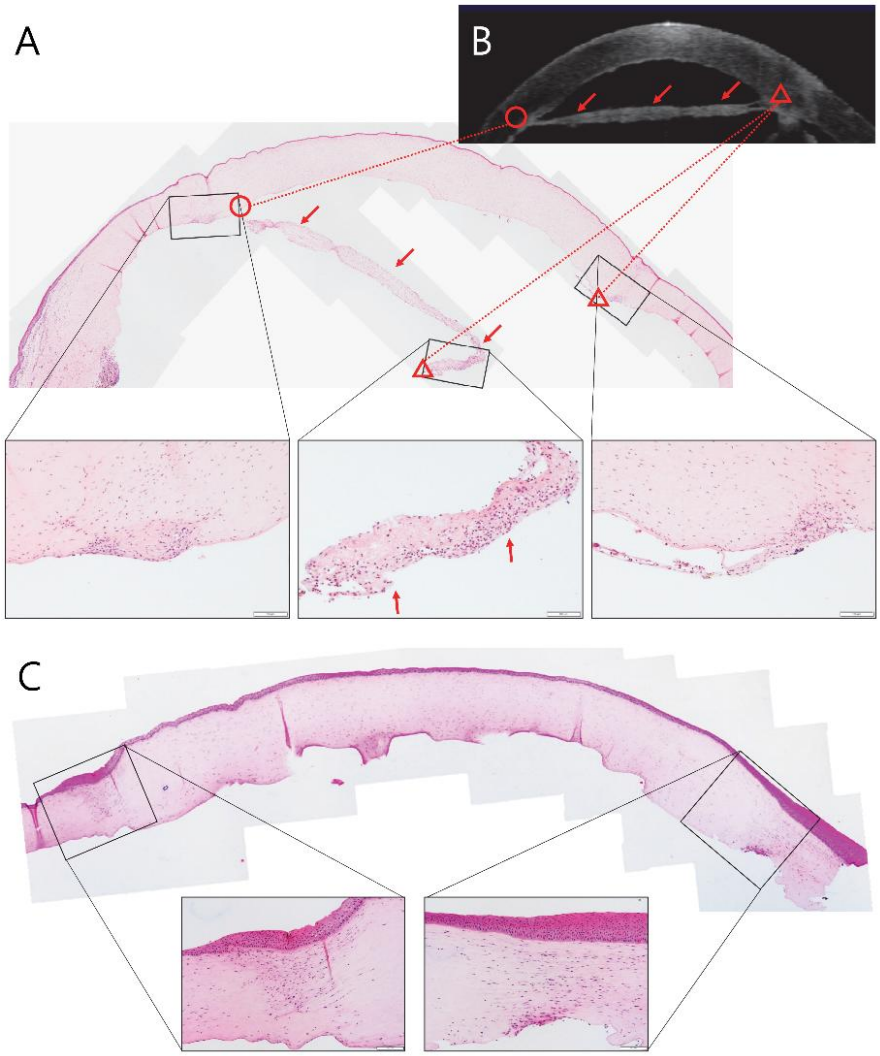


Figure 1.10. Representative hematoxylin & eosin staining of grafts. (A) Rejected graft as well as the retrocorneal membrane showed diffuse inflammatory cell infiltration (control group). (B) Retrocorneal membrane (red arrows) was well observed in anterior segment optical coherence tomography. The retrocorneal membrane was detached during the tissue staining process (hollow red triangle indicates that each edge of the retrocorneal membrane). (C) Surviving graft showed minimal infiltration of inflammatory cells limited to the donor-recipient junction (CD20 group).

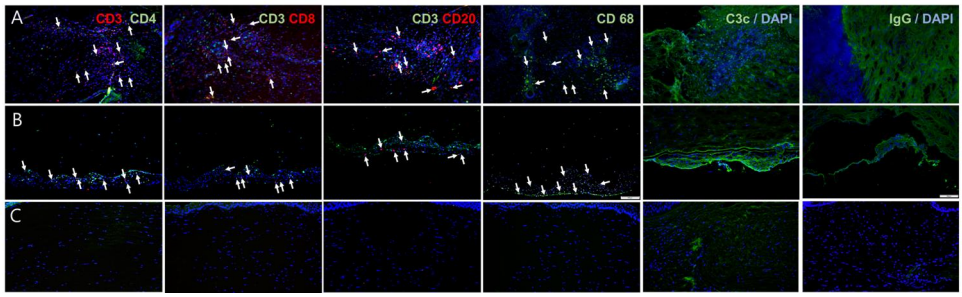


Figure 1.11. Immunofluorescence staining of rejected graft-recipient junction (A), retrocorneal membrane of rejected graft (endothelium side down; B), and surviving graft-recipient junction (C). Rejected graft showed dense infiltrating inflammatory cells at the graft junction (A) and retrocorneal membrane (B). There were also dense depositions of C3c and IgG in the rejected grafts. However, there were few staining of inflammatory cells, C3c, and IgG in a surviving graft (C). (magnification: × 200).

DISCUSSION

Our study reports that 1) GTKOm pig-to-NHP full thickness corneal xenotransplantation can attain successful graft survival time beyond 6 months, 2) anti-CD20 Ab treatment is required even when using GTKO grafts, and 3) graft survival is not dependent on the donor ages.

GTKO alone cannot overcome the rejection of corneal xenotransplantation. B cells were only inhibited with anti-CD20 Ab treatment. Because of GTKO, the level of anti-αGal Abs were not increased in any of the recipients, as reported in a previous study.[11] The degree of anti-non-gal Ab and extensive antigen-specific IgG deposits in rejected grafts corresponded with Chen's report which showed a correlation between anti-non-Gal Ab and humoral rejection after anti-αGal neutralization in kidney xenotransplantation.[22] These results suggest that anti-non-Gal Ab or other unknown antigens may play a role in GTKOm corneal graft survival.

Given that T cells are key players in allotransplantation and that rejected corneal xenografts demonstrated severe T cell infiltration,[23-25] T cell response must be suppressed in both WT and GTKOm corneal grafted NHPs. Tacrolimus was administered in both groups for this purpose. However, this study was not designed simultaneously. We first assessed the efficacy of GTKOm xenograft using minimal dosages of tacrolimus, and found this protocol was inadequate for the control of rejection. Thereafter, we evaluated the efficacy of GTKOm xenograft using a maximal dosage of tacrolimus along with anti-CD20 Ab application. The difference

in tacrolimus dose could have affected the survival outcome, but since the T cell profiles did not differ between the groups, the effect of inconsistent tacrolimus administration on the survival rates is considered to be minimal.

In this study, complement activation in the AH was associated with graft rejection, consistent with previous study finidings.[15,26] Transgenic pigs expressing human complement regulatory proteins (CRPs) show prolonged xenograft survival.[27] Thus, GTKO/CRP knock-in pigs may be promising for corneal xenotransplantation. We used TKOm or GTKO/hCD39 KIm pigs. The two recipients with TKOm corneas showed graft survival greater than 6 months. However, the advantage of TKOm over GTKOm is inconclusive, considering that rhesus macaques express N-glycolylneuraminic acid synthesized by the CMAH gene. Silencing the iGb3s gene does not appear to contribute to xenogeneic rejection.[28] Expression of hCD39, known to reduce platelet activation,[29] may contribute to graft survival.

Unlike WT grafted NHPs, retrocorneal membranes were frequently found in GTKOm grafted NHPs, which corresponded with previous study findings.[10,11] Retrocorneal membranes expressed CK8/18, α-SMA, and vimentin, indicating that they may originate from metaplastic endothelial cells (Figure 1.12).[10] Early formation of a thin membrane resolved, suggesting that inflammatory cells may be contributing to its presence. Aflibercept, known to reduce early frequency of immune cells, may be related to the resolution.[30] Conversely, formation of thick retrocorneal membranes in later stages were persistent, presumably because they may originate from metaplastic endothelial cells during inflammation.

Notably, corneal melt was frequently observed on the recipient side of the junction in GTKOm graft rejection cases (Figure 1.4). It was rarely observed in recipients with WT grafts.[15,16,18] Corneal melt is also rare in human allotransplantation. Other unknown xenoantigens may be hypersensitized to compensate for the reduction of αGal. Further study is warranted.

We evaluated corneal optical profiles and endothelial cell functions in GTKOm corneas and found that data of these cornea were comparable to the data in SNU WT miniature pig or human cornea (Table 1.1 and Figure 1.1).[21] This suggests that GTKOm donors are appropriate for corneal transplantation.

This study was limited in that 1) heterogeneously genetic modified pigs along with GTKO pigs were included, although the effect seems to be insignificant; 2) tacrolimus dosage was variable between those two groups, which may have affected the survival; 3) small sample size; and 4) the data of last examination was collected at the point of sudden death (POD 83) in one recipient with a surviving graft. Nonetheless, we believe our study is valuable because it demonstrates long-term graft survival in GTKOm pig-to-NHP full thickness corneal xenotransplantation when treated with anti-CD20 Ab.

In conclusion, GTKOm pig corneas are a practical substitute for human transplantation but require appropriate immunosuppression, including anti-CD20 Ab. GTKO alone is insufficient to reduce rejection, and inhibition of B cells and complement activation are required.

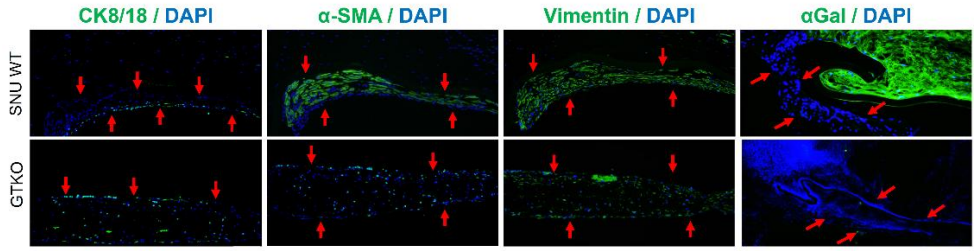


Figure 1.12. Immunofluorescence staining of retrocorneal membranes (red arrow) in SNU WT (upper) and GTKOm (lower) pig grafts showing positive stain for cytokeratin (CK8/18), α-smooth muscle (α-SMA), and vimentin, indicating that they may originate from metaplastic endothelial cells. In immunofluorescent staining for αGal, WT pig graft was positive. As expected, GTKOm pig graft was negative with αGal expression. None of the retrocorneal membranes of WT and GTKOm pig grafts expressed αGal, suggesting that it may be derived from the NHP cells (host) and not from pig cells (donor). (magnification × 200). SNU, Seoul National University; WT, wild type; GTKOm, α1,3-galactosyltransferase gene knockout miniature.

CHAPTER 2

Predictive biomarkers for graft rejection in pig-to-non-human primate corneal xenotransplantation

INTRODUCTION

Corneal xenotransplantation using pig donors has been investigated as a substitute for human donor corneas.[15,18,31,32] One of the challenges involving xenotransplantation relates to hyperacute rejection mediated by the natural antibody against Galalpha1,3Galbeta1,4GlcNAc-R (αGal) synthesized by α1,3-galactosyltransferase because non-human primates (NHPs) and humans carry natural anti-αGal IgM.[24] Corneas express αGal, although at lower levels than vascular endothelial cells do.[7] Further, the cornea is immune-privileged.[33-35] Pig-to-NHP corneal xenotransplantation has been reported to result in longer graft survival compared with other solid organ xenotransplantation.[4]

Although the success rate of low-risk corneal allotransplantation is greater than 90%,[34] the rejection rate can be up to 70% in high-risk cases.[36,37] In allograft rejection, both innate and adaptive immunities are involved.[38] Th1 cells play an important role.[23,39] In corneal xenotransplantation, the rejection process may evoke higher innate response in addition to T-cell responses.[24]

Several biomarkers are associated with corneal allograft rejection. Cytokines such as interleukin (IL)-6, IL-10, interferon (IFN)-γ, monocyte chemoattractant protein-1, and inflammatory monocytes in aqueous humor (AH), and impaired regulatory T cells have been reported in corneal allograft rejection.[39-42] In pig-to-NHP corneal xenotransplantation, the increase in IFN, tumor necrosis factor, IL-4, IL-5, IL-6, IL-10, and C3a levels was related to graft rejection.[11,43,44]

However, these biomarkers were based on a small population of subjects and no study analyzed the predictive ability of biomarkers in corneal xenotransplantation.

In this study, a retrospective analysis of various parameters was conducted to investigate the role of predictive biomarkers in graft rejection within 6 months in NHPs who underwent corneal xenotransplantation, using unpublished data or results of previous studies.[15,31,45]

MATERIALS AND METHODS

This study adhered to ARVO Statement regarding the Use of Animals in Ophthalmic and Vision Research. This study was approved by Seoul National University (SNU) (IACUC: SNU-151102-3) and SNU Hospital (IACUC: 09-0156, 11-0152, 12-0207, 13-0221, 15-0171).

Recipient characteristics and study design

Between 2010 and 2018, 38 rhesus macaques which had undergone full-thickness porcine corneal xenotransplantation were included.[15,31,45] Among them, four NHPs dying within 3 months without graft rejection were excluded. Donor pig characteristics, immunosuppressants used, and graft survival in all NHP recipients (n = 34) are summarized in Table 2.1. Briefly, 29 NHPs grafted with SNU wild-type (WT) miniature pig corneas reported previously,[15,31,45] and five NHPs grafted with α1,3-galactosyltransferase gene-knockout (GTKO, n = 4) or GTKO/human CD39 knockin (n = 1) miniature pig corneas in a new experiment were included.

Penetrating keratoplasty procedures were described previously.[15,31] NHPs were administered systemic and topical immunosuppressants listed in Table 2.1. Systemic immunosuppression was scheduled for six months. All NHPs received topical prednisolone acetate 1% (Pred forte®; Allergan, Irvine, CA, USA) daily for 3 months and injected subconjunctivally with dexamethasone 1.5 mg/0.3 mL (JW Pharmaceutical, Seoul, Republic of Korea) every week for 6 months.

Postoperative 2 or 4-week biomarkers for predicting graft rejection within 6 months were evaluated. To investigate 2 or 4-week biomarker candidates, 34 NHPs were divided into two groups: (a) graft rejection within 6 months (rejection group); and (b) graft survival until 6 months (survival group) (Table 2.2). The entire rejection group included all NHPs whose graft was rejected within a 6-month period, while late rejection group included NHPs whose graft was rejected after more than 4 weeks up to 6 months. None of the NHPs showed rejection at postoperative week 2. In the evaluation of the 2-week biomarkers, entire rejection group (n = 16) or late rejection group (n = 12) was compared to survival group (n = 18). In the evaluation of 4-week biomarkers, four NHPs showing rejection within 4 weeks were excluded and late rejection group (n = 12) was compared to survival group (n = 18).

To analyze 2 or 4-week biomarker candidates, blood or AH was collected to obtain the T and B cells, Abs, and C3a. Biomarker candidates were evaluated by comparing the rejection and survival groups at baseline, week 2, and week 4. AH C3a assay was performed only at week 4. In addition, we performed subgroup analysis to evaluate the effect of GTKO on predictive biomarkers. The subgroup analysis involved NHPs carrying rejected WT grafts and those carrying rejected GTKO grafts. Similar analysis was conducted to compare NHPs carrying surviving WT grafts and NHPs with rejected WT grafts after excluding those with GTKO xenografts. Receiver operating characteristic (ROC) curve analysis was performed to determine the predictive ability of the biomarkers, and areas under the curves (AUCs) were calculated to determine the level of discrimination.[46]

Graft score and definition of rejection

The corneal graft score (0-12) was calculated based on opacity, edema, and vascularization as described previously.[17] Scores ≥ 6 were defined as graft rejection. Success criteria for corneal xenograft are based on 6-month graft survival.[17] Therefore, data were analyzed up to 6 months.

T and B cell assays

Sub-populations of T cells ($CD28^+CD95^+$ central memory, $CD28^-CD95^+$ effector memory, and $CD4^+CD25^+Foxp3^+$ regulatory cells) and activated B cells ($CD3^-CD20^+CD28^+$) in blood were evaluated.[15,18] For extracellular surface staining, cell suspensions were incubated for 30 minutes at 4°C with fluorescein-conjugated mouse anti-human Abs as follows: CD3-FITC (1:40), CD4-FITC (1:200), CD8-PerCp-Cy5.5 (1:200), CD25-APC, CD28-APC (1:200), CD95-PE (1:200), and CD20-PE (1:200). For intracellular Ab staining, cell suspensions were incubated at 4°C with fluorescein-conjugated mouse anti-human Abs as follows: IFN-γ-PE (1:200, 30 minutes) and Foxp3-PE (1:200, 1 hour). Intracellular IFN-γ staining was performed after stimulation overnight with anti-CD3 Ab (2.5 μg/mL) and anti-CD28 Ab (0.25 μg/mL) in the presence of GolgiPlug (brefeldin A; 1 μL/1 mL). All Abs were purchased from eBioscience (San Diego, CA, USA), except CD3-FITC (from BD PharMingen, San Diego, CA, USA) and anti-CD3 Ab (U-CyTech, Utrecht, The Netherlands). Data were acquired using a FACSCanto flow cytometer (Becton-Dickinson, Mountain View, CA, USA) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA) (Figure 2.1). Data were presented as the

absolute number of cells per unit volume or percentage of peripheral blood mononuclear cells.

Antibody assay

Plasma anti-αGal IgM/G Abs were measured by ELISA as previously described.[19] Concentrations of anti-αGal Abs were expressed as artificial units (AU)/mL. Plasma levels of donor pig-specific (DS) IgM/G Abs were measured using flow cytometric cross-match technique using donor PBMCs as targeting cells.[32] Concentrations of DS Abs were semi-quantitatively expressed as mean fluorescence intensity (MFI).

Complement (C3a) assay

Levels of C3a in the plasma or in the AH were measured using the OptEIA™ Human C3a ELISA Kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's protocols. The upper detection limit of C3a concentration of AH was 25 ng/mL.

Statistical analysis

Normality was assessed by Shapiro-Wilk test. Independent continuous variables were compared using the Mann-Whitney U test or independent *t*-test. To determine the predictive ability of biomarkers, we performed ROC curve analysis. AUCs over 0.7, 0.8, or 0.9 were considered as acceptable, excellent, or outstanding

discrimination, respectively.[46] The value with the maximum Youden index (J = sensitivity + specificity − 1) was regarded as the optimal cut-off.[47] Mann-Whitney U test/independent *t*-test or ROC curve analysis was performed using SPSS v20.0 (SPSS Inc., Chicago, IL, USA) or the pROC package in R (V.3.5.0; R Foundation, Vienna, Austria), respectively. A *P* value of < 0.05 was considered statically significant.

Table 2.1. Systemic immunosuppressive regimen and corneal graft survival in NHPs (n = 34)

Group No.	Group	Systemic Immunosuppressive regimen	Subject number	Donor pig	Graft survival	Reported year
1	Conventional steroid	Methylprednisolone	3	WT	21, 28, 29	2015[15]
2	CD154	Anti-CD154 Ab IVIG Methylprednisolone	4	WT	>192, >243, 318, 933	2015[15]
3	CD40	Anti-CD40 Ab IVIG Methylprednisolone	6	WT	41, >196, >203, >273, >422, >511	2018[31]
4	CD20 (Full dose)	Anti-CD20 Ab[a] Tacrolimus[c] IVIG Basiliximab Methylprednisolone	6	WT	134,>184,>210,>260,297,>470	2018[31]
5	CD20 (Low dose)	Anti-CD20 Ab[b] Tacrolimus[d] IVIG Basiliximab Methylprednisolone	7	WT	56, 92, 162, >181, >182, >182,>198	2018[45]
6	Tacrolimus only	Tacrolimus[e] IVIG Basiliximab Methylprednisolone	5	GTKO (n = 4), GTKO/hCD39KI (n = 1)	37, 55, 72, 91, 165	Unpublished
			3	WT	29, 149, 161	2018[45]

Groups 1-6 with topical immunosuppressants: All NHPs received topical prednisolone acetate 1% (Pred forte®; Allergan, Irvine, CA, USA) daily for 3 months and injected subconjunctivally with dexamethasone 1.5 mg/0.3 mL (JW Pharmaceutical, Seoul, Republic of Korea) every week for 6 months.

Groups 1-6: Methylprednisolone was used with the same protocol in all groups. It was intramuscularly administered at an initial dose of 2 mg/kg/day and tapered over 5 weeks.

Groups 2-5: IVIG was used with the same protocol in groups 2-5. It was intravenously administered on preoperative day 1 and postoperative day 14 at a

dose of 1 g/kg.

Group 2: Recombinant anti-CD154 Ab (V-regions from mouse 5C8 clone; C-regions human IgG1k) was intravenously administered 15 to 19 times at a dose of 20 mg/kg. (Am J Transplant. 2015;15:628-641)

Group 3: A mouse-rhesus chimeric monoclonal anti-CD40 Ab (2C10R4, NIH Nonhuman Primate Reagent Resource) was intravenously administered 15 times at a dose of 30-50 mg/kg. (Am J Transplant. 2018;18:2330-2341.)

Groups 4 and 5: Anti-CD20 Ab (Rituximab; MabThera®, Hoffmann-La Roche, Basel, Switzerland) was intravenously administered at a dose of 20 mg/kg on postoperative days 0 and 7, and every 2[a] or 3[b] months. (Am J Transplant. 2018;18:2330-2341.; Xenotransplantation. 2018;25:e12442)

Groups 4-6: Tacrolimus (Prograf®; Astellas Pharma US, Deerfield, IL, USA) was intramuscularly administered twice daily at a dose of 0.05[c] or 0.035[e] mg/kg or at a dose of 0.05 mg/kg for 4 weeks followed by 0.035 mg/kg[d].

Groups 4-6: Basiliximab was intravenously administered at a dose of 0.3 mg/kg on postoperative days 0 and 4.

WT, wild type; Ab, antibody; IVIG, intravenous immunoglobulin; GTKO, alpha1,3-galactosyltransferase gene-knockout; hCD39KI, human CD39 knockin

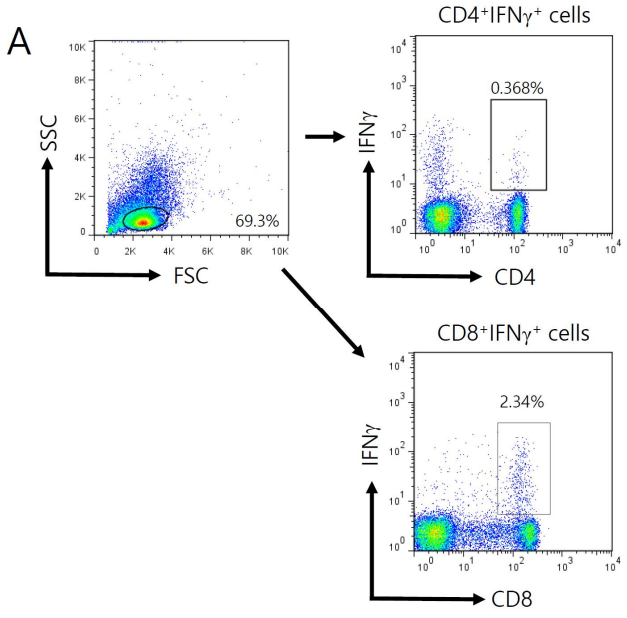
Table 2.2. Schematic of the study design for the 2 or 4-week biomarker candidates and group characteristics at weeks 2 and 4.

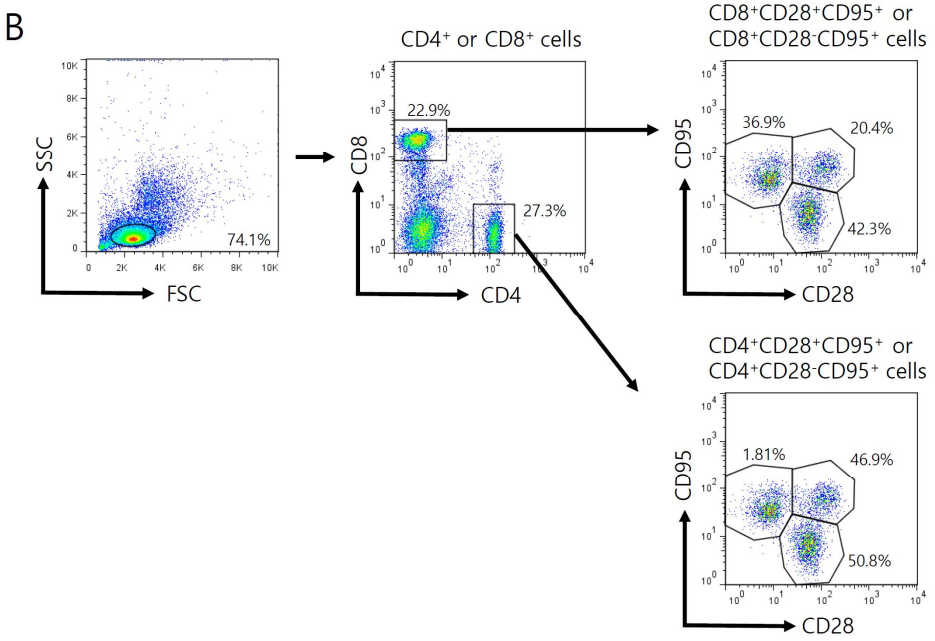
Analysis time	Biomarker candidates	Rejection group (number)	Average graft survival of each rejection group, mean ± SD (ranges)	Survival group number	Total number
Week 2	Blood: C3a, DS Abs, anti-αGal Abs, T and B cell subsets	Entire rejection* (16)	82.63 ± 54.38 (21~161)	18	34
		Late rejection† (12)	101.25 ± 50.15 (41~161)	18	30
Week 4	Blood: C3a, DS Abs, anti-αGal Abs, T and B cell subsets AH: C3a	Late rejection† (12)	101.25 ± 50.15 (41~161)	18	30

*named as "Entire rejection group". Entire rejection group includes NHPs whose grafts were rejected within 6 months.

†named as "Late rejection group". Late rejection group includes NHPs whose grafts were rejected at > week 4 up to month 6.

DS, donor pig-specific; Abs, antibodies; αGal, galactose-alpha-1,3-galactose; AH, aqueous humor


A
SSC
FSC
69.3%
CD4+IFNγ+ cells
IFNγ
CD4
0.368%
CD8+IFNγ+ cells
IFNγ
CD8
2.34%


B
SSC
FSC
74.1%
CD4+ or CD8+ cells
CD8
CD4
22.9%
27.3%
CD8+CD28+CD95+ or
CD8+CD28-CD95+ cells
CD95
CD28
36.9%
20.4%
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CD4+CD28+CD95+ or
CD4+CD28-CD95+ cells
CD95
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1.81%
46.9%
50.8%

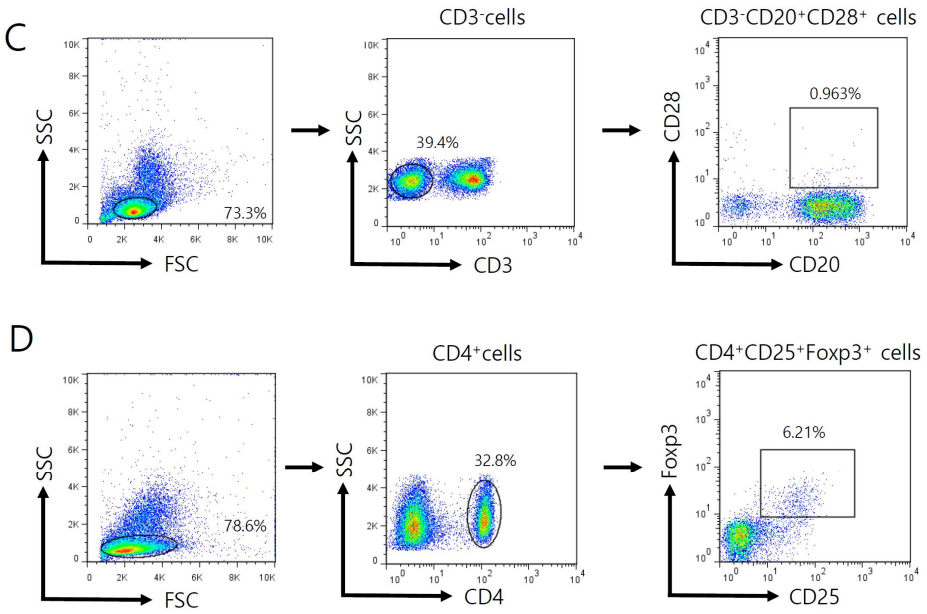

Figure 2.1. Representative multi-color flow cytometry gating strategies for IFNγ^+ $CD4^+$ or $CD8^+$ T cells (A), $CD28^+CD95^+$ central memory T cells, $CD28^-CD95^+$ effector memory T cells (B), $CD3^-CD20^+CD28^+$ activated B cells (C), and $CD4^+CD25^+Foxp3^+$ regulatory T cells (D).

RESULTS

Comparison of biomarker candidates in the rejection and survival groups

Baseline levels of biomarker candidates in the rejection and survival groups are shown in Table 2.3. Biomarker candidate levels at baseline did not significantly differ between the two groups.

At week 2, the graft score did not significantly differ between the groups (Table 2.4). Both the concentration and percentage of $CD8^+IFN\gamma^+$ cells at week 2 were significantly higher in the entire rejection group (52.32 ± 51.69 cells/mm^3 and 1.13 ± 1.16%, respectively) than in the survival group (17.68 ± 16.26 cells/mm^3 and 0.48 ± 0.56%, respectively; all $P = 0.032$). The difference was also significant when four NHPs showing rejection within 4 weeks were excluded. Both the concentration and percentage of $CD8^+IFN\gamma^+$ cells at week 4 and last examination showed no group-wise significant differences (Tables 2.4 and 2.5). The other biomarker candidates revealed no significant group-wise differences at week 2. At the last follow-up, the AH and plasma levels of C3a, DS IgG, and anti-αGal IgG were significantly higher in the entire rejection group than in the survival group.

The graft score at week 4 was not different between the groups (0.92 ± 1.56 and 0.22 ± 0.73, respectively; $P = 0.122$; Table 2.5). The AH C3a concentration at week 4 was significantly higher in the rejection group (16.56 ± 8.87 ng/mL) than in the survival group (6.25 ± 2.82 ng/mL; $P = 0.001$), and the other biomarker candidates did not differ between the groups. At the last follow-up,

the AH C3a and DS IgG concentrations were significantly higher in the late rejection group than in the survival group.

In subgroup analysis, the level of DS IgM was higher in the WT xenografted NHPs than in the GTKO xenografted NHPs throughout the follow-up. However, the DS IgG level was significantly higher in the WT xenografted NHPs at week 2 than in the GTKO xenografted NHPs without baseline differences (Table 2.6). Excluding GTKO xenografted NHPs, no significant differences in anti-αGal and DS Abs were found between the rejection and the survival groups (Table 2.7).

Predictability of presumptive biomarkers for graft rejection within 6 months

The $CD8^+IFN\gamma^+$ cells at week 2 and AH C3a at week 4 were presumptive biomarkers, which showed significant differences between the rejection and survival groups. The predictive abilities of these biomarkers were assessed (Figure 2.2).

The AUC of $CD8^+IFN\gamma^+$ cells at week 2 (both the concentration and percentage: 0.715; $P = 0.032$) showed acceptable discrimination ability for predicting rejection. The concentration of $CD8^+IFN\gamma^+$ cells estimated at 47.15 cells/mm^3 (sensitivity, 44%; and specificity, 94%) and the percentage of 0.56% (sensitivity, 69%; and specificity, 78%) represented the optimal cut-off values. In addition, the AUC of the AH C3a at week 4 (0.847; $P = 0.001$) showed excellent discrimination ability. AH C3a of 14.785 ng/mL (sensitivity, 58%; and specificity, 100%) was the best cut-off value. The positive and negative predictive values of AH C3a level of 14.785 ng/mL were 1.00 and 0.78, respectively, which indicates

that AH C3a concentration > 14.785 ng/mL at postoperative week 4 predicted rejection with a probability of 100%. Sensitivity, specificity, and positive and negative predictive values for each predictive biomarker are described in Table 2.8.

Table 2.3. Baseline levels of biomarker candidates in rejection and survival groups.

Biomarkers	Entire rejection (n = 16)	Late rejection (n = 12)	Survival (n = 18)	*P* (Entire rejection vs. Survival)	*P* (Late rejection vs. Survival)
AH C3a (ng/mL)	3.98 ± 1.74	3.87 ± 1.69	4.10 ± 1.09	0.810*	0.659*
Plasma C3a (ng/mL)	178.11 ± 130.47	125.80 ± 31.54	162.93 ± 128.36	0.308†	0.783†
DS IgG (MFI)	175.52 ± 229.95	99.28 ± 123.88	123.64 ± 208.17	0.905†	0.514†
DS IgM (MFI)	217.42 ± 318.36	130.79 ± 137.07	214.22 ± 127.15	0.190†	0.071†
Anti-αGal IgG (AU/mL)	102.33 ± 207.52	106.08 ± 236.04	61.84 ± 159.23	0.512†	0.799†
Anti-αGal IgM(AU/mL)	269.77 ± 243.15	281.14 ± 277.50	366.29 ± 202.75	0.053†	0.075†
$CD4^+IFN\gamma^+$ (cells/mm^3)	12.14 ± 16.74	11.75 ± 17.24	17.02 ± 22.16	0.352†	0.330†
$CD8^+IFN\gamma^+$ (cells/mm^3)	34.28 ± 30.39	35.25 ± 29.68	54.45 ± 51.71	0.317†	0.498†
$CD4^+$ CM (cells/mm^3)	329.44 ± 157.63	365.36 ± 154.82	287.15 ± 130.26	0.398*	0.146*
$CD4^+$ EM (cells/mm^3)	30.31 ± 32.82	35.68 ± 35.67	53.78 ± 63.30	0.370†	0.735†
$CD8^+$ CM (cells/mm^3)	130.52 ± 90.86	143.32 ± 95.86	110.71 ± 44.94	0.704†	0.421†
$CD8^+$ EM (cells/mm^3)	388.75 ± 239.71	373.99 ± 248.89	487.93 ± 319.19	0.408†	0.352†
ActB (cells/mm^3)	8.21 ± 6.58	9.78 ± 6.65	8.00 ± 6.83	0.945†	0.397†
Treg (cells/mm^3)	41.85 ± 29.41	49.66 ± 29.93	27.05 ± 24.07	0.112†	0.057†
$CD4^+IFN\gamma^+$ (%)	0.33 ± 0.47	0.30 ± 0.52	0.51 ± 0.70	0.334†	0.204*
$CD8^+IFN\gamma^+$ (%)	0.88 ± 0.79	0.82 ± 0.79	1.58 ± 1.62	0.157†	0.138*
$CD4^+$ CM (%)	7.75 ± 3.40	8.19 ± 3.75	8.29 ± 4.08	0.679*	0.945†
$CD4^+$ EM (%)	0.75 ± 0.90	0.84 ± 1.01	1.65 ± 2.24	0.301†	0.472*
$CD8^+$ CM (%)	3.00 ± 1.70	3.18 ± 1.90	3.19 ± 1.39	0.719*	0.988†
$CD8^+$ EM (%)	9.59 ± 5.47	8.30 ± 4.56	13.10 ± 7.96	0.138†	0.054*
ActB (%)	0.18 ± 0.16	0.22 ± 0.16	0.24 ± 0.22	0.469†	0.832*
Treg (%)	1.02 ± 0.59	1.10 ± 0.64	0.75 ± 0.60	0.195*	0.140†

Entire rejection group includes NHPs whose grafts were rejected within 6 months.

Late rejection group includes NHPs whose grafts were rejected at > week 4 up to month 6.

Data are presented as mean ± SD.

vs, versus; AH, aqueous humor; DS, donor pig-specific; αGal, galactose-alpha-1,3-galactose; IFN, interferon; CM, central memory T cells; EM, effector memory T cells; ActB, activated B cells; Treg, regulatory T cells

*Independent T-test (two-tailed), †Mann-Whitney U test (two-tailed)

Table 2.4. Values of biomarker candidates at postoperative week 2 in the rejection and survival groups.

Biomarkers	Week 2					Last FU		
	Entire rejection (n = 16)	Late rejection (n = 12)	Survival (n = 18)	*P* (Entire rejection vs. Survival)	*P* (Late rejection vs. Survival)	Entire rejection (n = 16)	Survival (n = 18)	*P*
Graft score	0.94 ± 1.73	0.25 ± 0.87	0.33 ± 1.41	0.145*	0.807*	6.56 ± 0.51	0.11 ± 0.47	< 0.001*
AH C3a (ng/mL)[a]	NA	NA	NA	NA	NA	22.01 ± 5.94	6.50 ± 5.69	< 0.001*
Plasma C3a (ng/mL)	167.83 ± 131.33	117.89 ± 20.87	137.67 ± 62.22	0.863*	0.310*	183.38 ± 97.42	124.06 ± 52.63	0.028*
DS IgG (MFI)	680.30 ± 804.89	351.18 ± 386.45	395.23 ± 349.09	0.748*	0.438*	1124.00 ± 1269.31	240.83 ± 439.75	< 0.001*
DS IgM (MFI)	299.66 ± 534.32	148.94 ± 136.07	224.14 ± 105.64	0.343*	0.118†	252.13 ± 278.58	245.18 ± 144.93	0.485*
Anti-αGal IgG (AU/mL)	234.64 ± 248.13	136.05 ± 157.64	107.31 ± 75.46	0.255*	0.966*	385.48 ± 677.19	51.99 ± 111.92	0.007*
Anti-αGal IgM (AU/mL)	416.53 ± 337.88	320.23 ± 278.28	396.11 ± 209.65	0.512*	0.117*	353.34 ± 220.04	349.73 ± 171.86	0.730*
$CD4^+IFN\gamma^+$ (cells/mm^3)	19.41 ± 31.94	21.12 ± 35.13	7.33 ± 10.52	0.098*	0.063*	8.19 ± 11.83	15.93 ± 24.74	0.350*
$CD8^+IFN\gamma^+$ (cells/mm^3)	**52.32 ± 51.69**	**58.60 ± 51.48**	**17.68 ± 16.26**	**0.032***	**0.006***	33.88 ± 28.99	58.08 ± 52.49	0.142*
$CD4^+$ CM (cells/mm^3)	397.38 ± 194.67	450.52 ± 183.92	394.60 ± 377.43	0.317*	0.090*	352.13 ± 156.95	268.24 ± 125.61	0.093†
$CD4^+$ EM (cells/mm^3)	40.38 ± 58.57	49.81 ± 65.13	25.04 ± 32.95	0.558*	0.189*	31.44 ± 35.26	71.96 ± 107.06	0.617*
$CD8^+$ CM (cells/mm^3)	133.02 ± 70.14	146.60 ± 63.10	138.69 ± 130.00	0.605*	0.220*	138.69 ± 94.06	109.26 ± 56.17	0.270†
$CD8^+$ EM (cells/mm^3)	307.89 ± 159.73	325.36 ± 163.20	261.28 ± 253.64	0.121*	0.075*	498.63 ± 521.24	515.40 ± 430.83	0.641*
ActB (cells/mm^3)	6.40 ± 5.76	6.47 ± 6.43	3.77 ± 6.02	0.073*	0.175*	6.88 ± 12.41	4.32 ± 5.68	0.794*
Treg (cells/mm^3)	33.08 ± 32.11	40.49 ± 33.80	45.21 ± 72.34	0.490*	0.204*	27.75 ± 18.14	21.80 ± 16.84	0.233*
$CD4^+IFN\gamma^+$ (%)	0.48 ± 0.85	0.45 ± 0.88	0.18 ± 0.25	0.112*	0.099*	0.32 ± 0.62	0.29 ± 0.33	0.388*
$CD8^+IFN\gamma^+$ (%)	**1.13 ± 1.16**	**1.04 ± 0.76**	**0.48 ± 0.56**	**0.032***	**0.010***	0.98 ± 1.18	1.41 ± 1.32	0.227*
$CD4^+$ CM (%)	8.30 ± 2.87	9.01 ± 2.72	9.69 ± 4.54	0.448*	0.643†	8.39 ± 2.93	6.80 ± 3.45	0.162†
$CD4^+$ EM (%)	0.79 ± 1.16	0.96 ± 1.30	0.66 ± 0.66	0.863*	0.767*	0.70 ± 0.55	1.29 ± 1.47	0.370*
$CD8^+$ CM (%)	2.79 ± 1.20	2.96 ± 1.15	3.26 ± 1.56	0.878†	0.573†	3.26 ± 1.97	2.77 ± 1.50	0.617*
$CD8^+$ EM (%)	7.14 ± 4.80	6.10 ± 1.64	6.35 ± 4.14	0.334*	0.290*	10.52 ± 6.96	12.51 ± 8.66	0.605*
ActB (%)	0.12 ± 0.10	0.14 ± 0.11	0.08 ± 0.09	0.138*	0.162*	0.13 ± 0.22	0.12 ± 0.17	0.822*
Treg (%)	0.81 ± 0.69	0.87 ± 0.63	0.92 ± 0.97	0.918*	0.611*	0.80 ± 0.48	0.67 ± 0.54	0.546*

Significant values at week 2 are shown in bold.

Entire rejection group includes NHPs whose grafts were rejected within 6 months.

Late rejection group includes NHPs whose grafts were rejected at > week 4 up to month 6.

Last FU; last follow-up; examination performed during the rejection period before sacrifice in the entire rejection group and at month 6 in the survival group.

[a]AH C3a assay was not performed at week 2 to avoid possible damage to the graft in the early postoperative period.

Data are presented as mean ± SD.

AH, aqueous humor; DS, donor pig-specific; αGal, galactose-alpha-1,3-galactose; IFN, interferon; CM, central memory T cells; EM, effector memory T cells; ActB, activated B cells; Treg, regulatory T cells

[*]Mann-Whitney U test (two-tailed), [†]Independent T-test (two-tailed).

Table 2.5. Values of biomarker candidates at postoperative week 4 in the late rejection and survival groups.

Biomarkers	Week 4			Last FU		
	Late rejection (n = 12)	Survival (n = 18)	*P*	Late rejection (n = 12)	Survival (n = 18)	*P*
Graft score	0.92 ± 1.56	0.22 ± 0.73	0.122*	6.25 ± 0.87	0.11 ± 0.47	< 0.001*
AH C3a (ng/mL)	**16.56 ± 8.87**	**6.25 ± 2.82**	**0.001***	22.84 ± 5.87	6.50 ± 5.69	< 0.001*
Plasma C3a (ng/mL)	136.58 ± 38.23	134.51 ± 90.86	0.122*	159.75 ± 46.15	124.06 ± 52.63	0.067†
DS IgG (MFI)	504.96 ± 684.17	337.04 ± 460.07	0.783*	585.91 ± 519.57	240.83 ± 439.75	0.001*
DS IgM (MFI)	182.66 ± 170.71	237.86 ± 105.88	0.477†	188.64 ± 164.677	245.18 ± 144.93	0.290*
Anti-αGal IgG (AU/mL)	112.82 ± 104.00	69.10 ± 81.70	0.057*	92.39 ± 115.24	51.99 ± 111.92	0.065*
Anti-αGal IgM (AU/mL)	337.70 ± 249.59	463.41 ± 320.68	0.138*	331.62 ± 246.16	349.73 ± 171.86	0.374*
$CD4^+IFN\gamma^+$ (cells/mm^3)	18.43 ± 29.32	9.03 ± 10.49	0.374*	7.58 ± 12.89	15.93 ± 24.74	0.268*
$CD8^+IFN\gamma^+$ (cells/mm^3)	60.23 ± 72.74	51.46 ± 68.79	0.352*	32.75 ± 30.14	58.08 ± 52.49	0.144*
$CD4^+$CM (cells/mm^3)	398.93 ± 187.66	305.11 ± 173.46	0.171†	357.83 ± 150.53	268.24 ± 125.61	0.088†
$CD4^+$EM (cells/mm^3)	79.95 ± 157.66	36.55 ± 45.12	0.498*	38.67 ± 38.15	71.96 ± 107.06	0.816*
$CD8^+$CM (cells/mm^3)	140.89 ± 66.14	105.92 ± 54.43	0.125†	140.67 ± 92.68	109.26 ± 56.17	0.256†
$CD8^+$EM (cells/mm^3)	557.01 ± 453.75	390.70 ± 305.89	0.253*	504.17 ± 567.70	515.40 ± 430.83	0.719*
ActB (cells/mm^3)	3.74 ± 3.46	2.82 ± 4.04	0.175*	4.42 ± 5.27	4.32 ± 5.68	0.966*
Treg (cells/mm^3)	41.51 ± 44.26	42.58 ± 56.44	0.949*	29.25 ± 18.40	21.80 ± 16.84	0.182*
$CD4^+IFN\gamma^+$ (%)	0.45 ± 0.93	0.25 ± 0.26	0.933*	0.27 ± 0.66	0.29 ± 0.33	0.189*
$CD8^+IFN\gamma^+$ (%)	1.23 ± 1.55	1.23 ± 1.50	0.657*	0.81 ± 1.08	1.41 ± 1.32	0.138*
$CD4^+$ CM (%)	8.21 ± 2.70	8.82 ± 3.55	0.866*	8.38 ± 3.05	6.80 ± 3.45	0.209†
$CD4^+$ EM (%)	1.43 ± 2.30	1.02 ± 0.95	0.672*	0.83 ± 0.58	1.29 ± 1.47	0.703*
$CD8^+$ CM (%)	3.06 ± 1.53	3.23 ± 1.23	0.611*	3.39 ± 2.22	2.77 ± 1.50	0.366†
$CD8^+$ EM (%)	11.18 ± 6.52	11.12 ± 5.55	0.882*	10.57 ± 7.07	12.51 ± 8.66	0.582*
ActB (%)	0.08 ± 0.08	0.10 ± 0.12	0.703*	0.09 ± 0.09	0.12 ± 0.17	0.916*
Treg (%)	0.89 ± 0.78	1.25 ± 1.16	0.421*	0.82 ± 0.52	0.67 ± 0.54	0.512*

Significant values at week 4 are shown in bold.

Last FU; last follow-up; examination performed during the rejection period before sacrifice in the entire rejection group and at month 6 in the survival group.

Late rejection group includes NHPs whose grafts were rejected at > week 4 up to month 6.

Data are presented as mean ± SD.

AH, aqueous humor; DS, donor pig-specific; αGal, galactose-alpha-1,3-galactose; IFN, interferon; CM, central memory T cells; EM, effector memory T cells; ActB, activated B cells; Treg, regulatory T cells

*Mann-Whitney U test (two-tailed), †Independent T-test (two-tailed).

Table 2.6. Subgroup analysis showed differences in DS IgG and IgM levels in the rejection group according to donor pig type. The DS IgM was higher in the WT xenografted NHPs than in the GTKO xenografted NHPs throughout the follow-up, which was not clinically relevant. However, DS IgG was significantly higher in the WT xenografted NHPs at week 2 than in the GTKO xenografted NHPs without baseline differences, suggesting a possible association between the DS IgG level and rejection in the WT xenografted NHPs.

Biomarkers	Baseline			Week 2			Week 4			Last FU		
	WT (n = 11)	GTKO (n = 5)	*P**	WT (n = 11)	GTKO (n = 5)	*P**	WT (n = 7)	GTKO (n = 5)	*P**	WT (n = 11)	GTKO (n = 5)	*P**
AH C3a (ng/mL)	3.96 ± 1.48	4.00 ± 2.38	0.713	NA	NA	**NA**	14.19 ± 10.14	19.88 ± 6.20	0.508	20.51 ± 6.88	25.00 ± 0.00	0.116
Plasma C3a (ng/mL)	198.71 ± 153.21	132.80 ± 37.64	0.865	193.12 ± 153.37	112.20 ± 15.72	0.234	138.13 ± 49.92	134.40 ± 16.44	0.808	194.18 ± 112.73	159.60 ± 52.80	0.734
DS IgG (MFI)	234.73 ± 250.48	27.50 ± 4.36	0.157	915.14 ± 824.28	34.50 ± 14.73	**0.004**	694.51 ± 792.01	239.60 ± 442.05	0.062	1219.91 ± 1436.50	860.25 ± 716.81	0.896
DS IgM (MFI)	298.49 ± 347.61	14.75 ± 5.19	**0.005**	400.26 ± 598.23	23.00 ± 16.04	**0.004**	275.11 ± 145.20	20.88 ± 11.56	**0.008**	334.27 ± 284.22	26.25 ± 11.15	**0.004**
Anti-αGal IgG (AU/mL)	59.11 ± 79.90	197.40 ± 359.30	0.610	274.93 ± 257.86	146.00 ± 224.40	0.157	87.62 ± 68.43	148.10 ± 141.60	0.223	539.51 ± 776.69	46.60 ± 52.78	0.079
Anti-αGal IgM (AU/mL)	290.84 ± 286.04	223.40 ± 115.11	0.955	485.14 ± 386.37	265.60 ± 116.10	0.336	382.91 ± 318.97	274.40 ± 100.58	0.935	380.68 ± 242.14	293.20 ± 168.56	0.533
$CD4^+IFN\gamma^+$ (cells/mm^3)	8.77 ± 11.35	19.55 ± 25.07	0.193	13.16 ± 19.78	33.16 ± 50.05	0.062	14.80 ± 24.98	23.51 ± 37.04	0.372	5.36 ± 6.50	14.40 ± 18.69	0.211
$CD8^+IFN\gamma^+$ (cells/mm^3)	22.58 ± 23.35	60.02 ± 29.92	**0.008**	38.98 ± 38.42	86.07 ± 58.80	0.123	63.06 ± 87.29	56.26 ± 55.59	0.685	29.55 ± 30.17	43.40 ± 26.67	0.335
$CD4^+$ CM (cells/mm^3)	259.07 ± 137.95	484.26 ± 45.03	**0.004**	364.05 ± 178.08	470.70 ± 230.35	0.336	388.50 ± 202.00	413.53 ± 187.57	0.685	335.55 ± 166.87	388.60 ± 142.62	0.533
$CD4^+$ EM (cells/mm^3)	19.26 ± 23.06	54.63 ± 40.44	**0.047**	34.39 ± 63.54	53.57 ± 49.57	0.079	96.84 ± 206.41	56.32 ± 57.03	0.372	29.64 ± 40.62	35.40 ± 22.56	0.100
$CD8^+$ CM (cells/mm^3)	95.76 ± 58.61	207.00 ± 108.34	**0.036**	112.62 ± 59.05	177.90 ± 77.90	0.100	124.00 ± 67.65	164.54 ± 63.00	0.223	126.18 ± 84.86	166.20 ± 117.44	0.461
$CD8^+$ EM (cells/mm^3)	334.19 ± 200.55	508.76 ± 297.89	0.157	261.29 ± 125.24	410.41 ± 193.26	0.157	631.32 ± 594.31	452.98 ± 114.83	0.935	543.36 ± 618.41	400.20 ± 212.52	0.865
ActB (cells/mm^3)	6.79 ± 6.65	11.31 ± 5.85	0.193	4.81 ± 4.11	9.90 ± 7.74	0.192	3.98 ± 4.32	3.40 ± 2.18	0.808	7.27 ± 14.91	6.00 ± 4.53	0.153
Treg (cells/mm^3)	26.71 ± 18.23	75.15 ± 19.87	**0.003**	34.81 ± 37.46	29.27 ± 18.24	0.777	46.20 ± 47.11	34.95 ± 44.34	0.685	23.91 ± 13.32	36.20 ± 25.70	0.335
$CD4^+IFN\gamma^+$ (%)	0.25 ± 0.26	0.50 ± 0.78	0.533	0.35 ± 0.57	0.74 ± 1.33	0.335	0.23 ± 0.29	0.76 ± 1.44	0.935	0.22 ± 0.37	0.55 ± 1.01	0.496
$CD8^+IFN\gamma^+$ (%)	0.67 ± 0.62	1.33 ± 1.01	0.126	0.79 ± 0.46	1.40 ± 1.00	0.291	1.02 ± 1.04	1.52 ± 2.18	0.935	0.84 ± 1.00	1.28 ± 1.60	0.692
$CD4^+$ CM (%)	6.82 ± 3.42	9.80 ± 2.53	0.062	8.76 ± 3.29	7.30 ± 1.46	0.282	8.33 ± 2.92	8.03 ± 2.68	0.807	8.59 ± 3.26	7.94 ± 2.29	0.692
$CD4^+$ EM (%)	0.51 ± 0.53	1.28 ± 1.36	0.126	0.65 ± 1.08	1.08 ± 1.41	0.126	1.34 ± 2.47	1.55 ± 2.31	0.685	0.63 ± 0.49	0.86 ± 0.69	0.692
$CD8^+$ CM (%)	2.44 ± 1.17	4.22 ± 2.16	0.157	2.74 ± 1.30	2.89 ± 1.07	0.692	2.73 ± 1.28	3.53 ± 1.87	0.465	3.06 ± 1.76	3.71 ± 2.56	0.955
$CD8^+$ EM (%)	9.30 ± 5.62	10.23 ± 5.70	0.777	7.46 ± 5.71	6.44 ± 2.02	0.865	11.69 ± 6.78	10.48 ± 6.86	0.465	11.50 ± 7.93	8.36 ± 4.01	0.396
ActB (%)	0.16 ± 0.18	0.22 ± 0.11	0.234	0.11 ± 0.11	0.15 ± 0.09	0.461	0.10 ± 0.11	0.06 ± 0.03	0.808	0.14 ± 0.26	0.11 ± 0.08	0.193

Treg (%)	0.80 ± 0.52	1.51 ± 0.46	**0.027**	0.93 ± 0.76	0.56 ± 0.50	0.533	0.99 ± 0.83	0.74 ± 0.77	0.465	0.81 ± 0.51	0.77 ± 0.47	0.777

Significant P values are shown in bold.

Last FU; last follow-up; examination performed during the rejection period before sacrifice in the entire rejection group and at month 6 in the survival group.

Data are presented as mean ± SD.

DS, donor pig-specific; WT, wild type; GTKO, α-1,3-galactosyltransferase gene knockout; NHPs, non-human primates; AH, aqueous humor; NA, not available; αGal, galactose-alpha-1,3-galactose; IFN, interferon; CM, central memory T cells; EM, effector memory T cells; ActB, activated B cells; Treg, regulatory T cells

*Mann-Whitney U test (two-tailed)

Table 2.7. Subgroup analysis was performed to determine whether the inclusion of GTKO xenografted NHPs affected the anti-αGal or DS Abs as a biomarker. This analysis excluded GTKO xenografted NHPs from the rejection groups, because survival group did not include GTKO xenografted NHPs. There was no significant difference between the rejection and survival groups in the levels of anti-αGal and DS Abs.

	Entire rejection (n = 11) w/o GTKO	Survival (n = 18)	*P*
Baseline			
Donor specific IgG (AU/mL)	234.73 ± 250.48	123.64 ± 208.17	0.269*
Donor specific IgM (AU/mL)	298.49 ± 347.61	214.22 ± 127.15	0.880*
Anti-αGal IgG (AU/mL)	59.11 ± 79.90	61.84 ± 159.23	0.653*
Anti-αGal IgM (AU/mL)	290.84 ± 268.04	366.29 ± 202.75	0.116*
Week 2			
Donor specific IgG (AU/mL)	915.14 ± 824.28	395.23 ± 349.09	0.051*
Donor specific IgM (AU/mL)	400.26 ± 598.23	224.14 ± 105.64	0.693*
Anti-αGal IgG (AU/mL)	274.93 ± 257.87	107.31 ± 75.46	0.059†
Anti-αGal IgM (AU/mL)	485.14 ± 386.37	396.11 ± 209.65	0.964*
	Late rejection (n = 7) w/o GTKO	Survival (n = 18)	*P*
Week 4			
Donor specific IgG (AU/mL)	694.51 ± 792.01	337.04 ± 460.07	0.069*
Donor specific IgM (AU/mL)	275.11 ± 145.20	237.86 ± 105.88	0.593*
Anti-αGal IgG (AU/mL)	87.62 ± 68.43	69.10 ± 81.70	0.226*
Anti-αGal IgM (AU/mL)	382.91 ± 318.97	463.41 ± 320.68	0.276*

Entire rejection group includes wild type xenografted NHPs whose grafts were rejected within 6 months.

Late rejection group includes wild type xenografted NHPs whose grafts were rejected at > week 4 up to month 6.

Data are presented as mean ± SD.

w/o, without; GTKO, α1,3-galactosyltransferase gene-knockout; NHP, non-human primate; αGal, galactose-alpha-1,3-galactose; DS, donor pig-specific; w/o GTKO, without GTKO xenografted NHPs;

*Mann-Whitney U test (two-tailed), †Independent T-test (two-tailed).

Table 2.8. Sensitivity, specificity, positive predictive value, and negative predictive values for each predictive biomarker.

$CD8^+IFN\gamma^+$ at week 2 (cells/mm^3)					$CD8^+IFN\gamma^+$ at week 2 (%)					AH C3a at week 4 (ng/mL)				
Value	Sensitivity	Specificity	PPV	NPV	Value	Sensitivity	Specificity	PPV	NPV	Value	Sensitivity	Specificity	PPV	NPV
0.00	1.00	0.00	0.47	NA	0.00	1.00	0.00	0.47	NA	0.000	1.00	0.00	0.40	NA
0.58	0.94	0.00	0.45	0.00	0.02	0.94	0.00	0.45	0.00	2.385	1.00	0.06	0.41	1.00
1.81	0.94	0.06	0.47	0.50	0.06	0.94	0.06	0.47	0.50	3.885	1.00	0.11	0.43	1.00
2.50	0.94	0.11	0.48	0.67	0.09	0.94	0.11	0.48	0.67	4.315	1.00	0.22	0.46	1.00
3.12	0.94	0.17	0.50	0.75	0.11	0.94	0.17	0.50	0.75	4.661	1.00	0.28	0.48	1.00
4.08	0.94	0.22	0.52	0.80	0.12	0.81	0.22	0.48	0.57	4.771	1.00	0.33	0.50	1.00
4.77	0.94	0.28	0.54	0.83	0.14	0.81	0.28	0.50	0.63	4.875	1.00	0.39	0.52	1.00
5.72	0.94	0.33	0.56	0.86	0.17	0.81	0.33	0.52	0.67	5.050	0.92	0.39	0.50	0.88
6.41	0.88	0.33	0.54	0.75	0.22	0.81	0.44	0.57	0.73	5.285	0.92	0.44	0.52	0.89
6.65	0.81	0.33	0.52	0.67	0.30	0.81	0.50	0.59	0.75	5.535	0.92	0.50	0.55	0.90
6.85	0.81	0.39	0.54	0.70	0.38	0.81	0.56	0.62	0.77	5.850	0.83	0.50	0.53	0.82
8.88	0.81	0.44	0.57	0.73	0.41	0.81	0.61	0.65	0.79	6.020	0.83	0.56	0.56	0.83
11.46	0.81	0.50	0.59	0.75	0.43	0.75	0.61	0.63	0.73	6.095	0.83	0.61	0.59	0.85
12.91	0.75	0.50	0.57	0.69	0.45	0.69	0.61	0.61	0.69	6.275	0.75	0.61	0.56	0.79
14.44	0.69	0.50	0.55	0.64	0.48	0.69	0.67	0.65	0.71	6.755	0.75	0.67	0.60	0.80
15.33	0.69	0.56	0.58	0.67	0.51	0.69	0.72	0.69	0.72	7.155	0.75	0.72	0.64	0.81
16.23	0.69	0.61	0.61	0.69	**0.56**	**0.69**	**0.78**	**0.73**	**0.74**	7.395	0.75	0.78	0.69	0.82
18.73	0.63	0.61	0.59	0.65	0.64	0.63	0.78	0.71	0.70	8.300	0.67	0.78	0.67	0.78
20.79	0.63	0.67	0.63	0.67	0.72	0.63	0.83	0.77	0.71	9.305	0.67	0.83	0.73	0.79
21.01	0.63	0.72	0.67	0.68	0.78	0.56	0.83	0.75	0.68	9.850	0.67	0.89	0.80	0.80
22.75	0.56	0.72	0.64	0.65	0.82	0.56	0.89	0.82	0.70	10.805	0.58	0.89	0.78	0.76
25.22	0.50	0.72	0.62	0.62	0.85	0.50	0.89	0.80	0.67	11.790	0.58	0.94	0.88	0.77
26.38	0.50	0.78	0.67	0.64	0.89	0.44	0.89	0.78	0.64	**14.785**	**0.58**	**1.00**	**1.00**	**0.78**
32.38	0.44	0.78	0.64	0.61	0.98	0.38	0.89	0.75	0.62	20.000	0.50	1.00	1.00	0.75
40.83	0.44	0.83	0.70	0.63	1.18	0.31	0.89	0.71	0.59	23.400	0.42	1.00	1.00	0.72
43.81	0.44	0.89	0.78	0.64	1.36	0.25	0.89	0.67	0.57	24.650	0.33	1.00	1.00	0.69
47.15	**0.44**	**0.94**	**0.88**	**0.65**	1.55	0.19	0.89	0.60	0.55	26.000	0.00	1.00	NA	0.60
50.39	0.38	0.94	0.86	0.63	1.74	0.19	0.94	0.75	0.57					
61.31	0.38	1.00	1.00	0.64	1.95	0.13	0.94	0.67	0.55					
73.40	0.31	1.00	1.00	0.62	2.54	0.13	1.00	1.00	0.56					
92.29	0.25	1.00	1.00	0.60	3.72	0.06	1.00	1.00	0.55					
112.71	0.19	1.00	1.00	0.58	5.48	0.00	1.00	NA	0.53					
115.45	0.13	1.00	1.00	0.56										
143.40	0.06	1.00	1.00	0.55										
172.33	0.00	1.00	NA	0.53										

Bold text represents values at optimal cut-off.

IFN, interferon, AH, aqueous humor; PPV, positive predictive value; NPV, negative predictive value; NA, not available

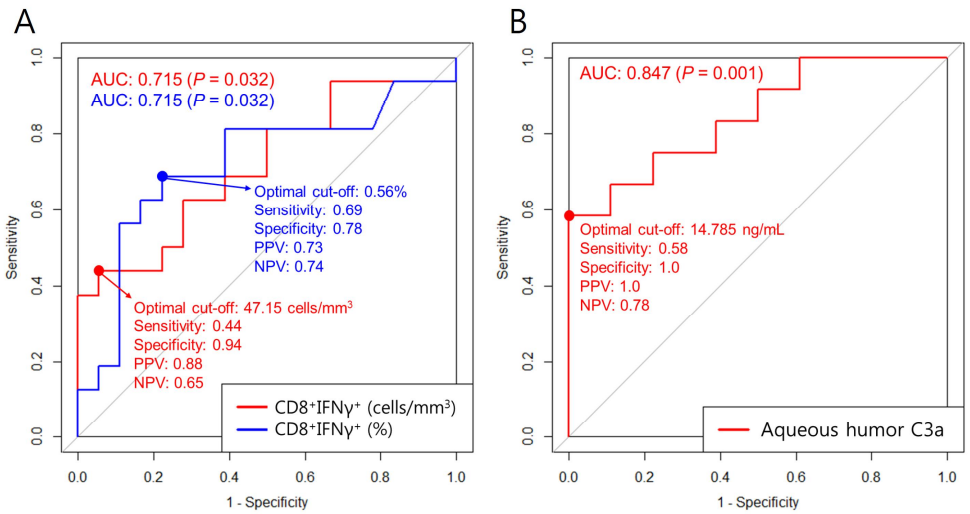

Figure 2.2. Receiver operator characteristic curve analysis of $CD8^{+}IFN\gamma^{+}$ at week 2 (A) and aqueous humor C3a at week 4 (B) for predicting graft rejection. (A) The area under the curve (AUC) of the $CD8^{+}IFN\gamma^{+}$ cells at week 2 was 0.715 (both concentration and percentage), indicating acceptable discrimination ability. The $CD8^{+}IFN\gamma^{+}$ cell concentration of 47.15 cells/mm^3 (sensitivity, 44%; and specificity, 94%) and percentage of 0.56% (sensitivity, 69%; and specificity, 78%) were the best cut-off values. (B) The AUC of the AH C3a at week 4 was 0.847, indicating excellent discrimination ability. The AH C3a level of 14.785 ng/mL (sensitivity, 0.58%; and specificity, 100%) represented the best cut-off value. Positive and negative predictive values of AH C3a of 14.785 ng/mL were 1.00 and 0.78, respectively. Round dot denotes the optimal cut-off value. AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value

DISCUSSION

Corneal xenograft rejection is mediated by both innate and adaptive immune systems. The innate immune response is immediate, while the adapted immune response occurs within several days or weeks.[48] As shown in Tables 2.1 and 2.2, rejection in corneal xenotransplantation occurred frequently between months 1 and 3. The graft scores were similar between the two groups at weeks 2 and 4, which indicates that changes in predictive biomarker levels precede corneal morphological changes during the rejection process. This finding suggests the clinical relevance of predictive biomarkers for the detection of rejection earlier than slit-lamp microscopy. Therefore, our study showed that the 2 or 4-week predictive biomarker profiles may facilitate early intervention against rejection. In this study, the levels of $CD8^{+}IFN\gamma^{+}$ cells at week 2 and AH C3a at week 4 were significantly higher in the rejection group than in the survival group and showed acceptable or excellent discrimination abilities for predicting rejection within 6 months.

In contrast to solid organ transplantation, corneal graft rejection can be detected by slit lamp examination. However, at early stages of immune reaction, the cornea may retain transparency, which may contribute to detection failure of early rejection.[49] Corneal edema can be reversed upon early detection of rejection and appropriate management before irreversible graft failure occurs.[50] In this regard, the changes in $CD8^{+}IFN\gamma^{+}$ cells may represent a key 2-week biomarker for the early detection of rejection. At the last follow-up, no systemic differences in $CD8^{+}IFN\gamma^{+}$ cells were found, which may be explained by the localization of cells

in the cornea, a finding supported by previous studies showing infiltration of $CD8^+$ cells in rejected grafts.[15,18,32,51]

AH complement activation is related to both innate and adaptive immunity.[52] Our previous studies indicated the presence of C3c deposits as well as high levels of AH C3a in NHPs with rejected grafts, but rarely in NHPs with surviving grafts.[15,18,31,32] The combined data suggest that AH complement is a critical factor for rejection. The AH C3a assay was performed at postoperative week 4 to avoid possible graft damage in the early period. Therefore, further studies are needed to investigate the potential role of AH C3a as a 2-week biomarker.

We are planning a clinical trial of corneal xenotransplantation.[53] The results obtained in this study will be used as a standard of reference to predict rejection in the clinical trial. In particular, our results indicated that AH C3a is a potentially critical biomarker with a positive predictive value of 1.0 at the optimal cut-off value. In our study, no complications occurred during AH collection,[15,18,31,32] which is considered as a routine procedure for patients undergoing PCR testing for virus,[54] and can be performed safely with adequate precaution.[55]

DS IgG and anti-αGal IgG were not significant predictors of rejection. In WT pig-to-NHPs corneal xenotransplantation, high levels of αGal epitope or IgG deposits are present in the rejected graft.[15,18,31] Therefore, subgroup analysis was performed to determine whether the inclusion of GTKO porcine corneal grafts in NHPs affected the changes in DS or anti-αGal Abs as biomarkers (Tables 2.6 and 2.7). The level of DS IgM was higher in WT xenografted NHPs than in GTKO

xenografted NHPs during the follow-up, which was not clinically relevant. However, DS IgG was significantly higher in WT xenografted NHPs at week 2 than in GTKO xenografted NHPs without significant baseline differences, suggesting a possible association between the DS IgG level and rejection in WT xenografted NHPs. As shown in Table 2.7, subgroup analysis was performed after excluding GTKO xenografted NHPs from the rejection groups (11 in entire rejection / 7 in late rejection), because the survival group did not include GTKO xenografted NHPs. Although no significant differences in anti-αGal and DS Abs were found between the rejection and survival groups, we observed changes in DS IgG at week 2 in the rejection group, suggesting that the inclusion of GTKO xenografted NHPs might alter the DS IgG biomarker levels. Therefore, our study limitation related to inclusion of both WT and GTKO donor grafts. Another limitation involved inclusion of NHPs under various immunosuppression regimens. Heterogeneous immunosuppressants exhibit varied effects on the immune response. Further biomarker studies including homogeneous optimal donors and immunosuppressant types are needed.

In conclusion, $CD8^{+}IFN\gamma^{+}$ cells at week 2 and AH C3a concentrations at week 4 showed potential as useful biomarkers for predicting graft rejection in pig-to-NHP corneal xenotransplantation. Those biomarkers may be used as a standard of reference to predict rejection in clinical trials of corneal xenotransplantation. To the best of our knowledge, this study is the first to report predictive biomarkers for graft rejection in corneal xenotransplantation.

REFERENCES

1. Tan DT, Dart JK, Holland EJ, et al. Corneal transplantation. *Lancet.* 2012;379(9827):1749-1761.
2. Hara H, Cooper DK. Xenotransplantation--the future of corneal transplantation? *Cornea.* 2011;30(4):371-378.
3. Kim MK, Wee WR, Park CG, et al. Xenocorneal transplantation. *Current opinion in organ transplantation.* 2011;16(2):231-236.
4. Kim MK, Hara H. Current status of corneal xenotransplantation. *Int J Surg.* 2015;23(Pt B):255-260.
5. Yamada K, Yazawa K, Shimizu A, et al. Marked prolongation of porcine renal xenograft survival in baboons through the use of alpha1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. *Nat Med.* 2005;11(1):32-34.
6. Kuwaki K, Tseng YL, Dor FJ, et al. Heart transplantation in baboons using alpha1,3-galactosyltransferase gene-knockout pigs as donors: initial experience. *Nat Med.* 2005;11(1):29-31.
7. Lee HI, Kim MK, Oh JY, et al. Gal alpha(1-3)Gal expression of the cornea in vitro, in vivo and in xenotransplantation. *Xenotransplantation.* 2007;14(6):612-618.
8. Choi HJ, Kim MK, Lee HJ, et al. Effect of alphaGal on corneal xenotransplantation in a mouse model. *Xenotransplantation.* 2011;18(3):176-182.
9. Cooper DK, Dorling A, Pierson RN, 3rd, et al. Alpha1,3-

galactosyltransferase gene-knockout pigs for xenotransplantation: where do we go from here? *Transplantation.* 2007;84(1):1-7.

10. Lee W, Mammen A, Dhaliwal DK, et al. Development of retrocorneal membrane following pig-to-monkey penetrating keratoplasty. *Xenotransplantation.* 2017;24(1).

11. Dong X, Hara H, Wang Y, et al. Initial study of alpha1,3-galactosyltransferase gene-knockout/CD46 pig full-thickness corneal xenografts in rhesus monkeys. *Xenotransplantation.* 2017;24(1).

12. Vabres B, Le Bas-Bernardet S, Riochet D, et al. hCTLA4-Ig transgene expression in keratocytes modulates rejection of corneal xenografts in a pig to non-human primate anterior lamellar keratoplasty model. *Xenotransplantation.* 2014;21(5):431-443.

13. Ock SA, Lee J, Oh KB, et al. Molecular immunology profiles of monkeys following xenografting with the islets and heart of alpha-1,3-galactosyltransferase knockout pigs. *Xenotransplantation.* 2016;23(5):357-369.

14. Choi K, Shim J, Ko N, et al. Production of heterozygous alpha 1,3-galactosyltransferase (GGTA1) knock-out transgenic miniature pigs expressing human CD39. *Transgenic Res.* 2017;26(2):209-224.

15. Choi HJ, Lee JJ, Kim DH, et al. Blockade of CD40-CD154 costimulatory pathway promotes long-term survival of full-thickness porcine corneal grafts in nonhuman primates: clinically applicable xenocorneal transplantation. *Am J Transplant.* 2015;15(3):628-641.

16. Kim J, Choi SH, Lee HJ, et al. Comparative efficacy of anti-CD40

antibody-mediated co-stimulation blockade on long-term survival of full-thickness porcine corneal grafts in nonhuman primates. *Am J Transplant.* 2018.

17. Kim MK, Choi HJ, Kwon I, et al. The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of xenocorneal transplantation. *Xenotransplantation.* 2014;21(5):420-430.

18. Kim J, Kim DH, Choi HJ, et al. Anti-CD40 antibody-mediated costimulation blockade promotes long-term survival of deep-lamellar porcine corneal grafts in non-human primates. *Xenotransplantation.* 2017;24(3).

19. Kang HJ, Lee H, Park EM, et al. Increase in anti-Gal IgM level is associated with early graft failure in intraportal porcine islet xenotransplantation. *Ann Lab Med.* 2015;35(6):611-617.

20. Kang HJ, Lee H, Park EM, et al. Dissociation between anti-porcine albumin and anti-Gal antibody responses in non-human primate recipients of intraportal porcine islet transplantation. *Xenotransplantation.* 2015;22(2):124-134.

21. Kim DH, Kim J, Jeong HJ, et al. Biophysico-functional compatibility of Seoul National University (SNU) miniature pig cornea as xenocorneal graft for the use of human clinical trial. *Xenotransplantation.* 2016;23(3):202-210.

22. Chen G, Sun H, Yang H, et al. The role of anti-non-Gal antibodies in the development of acute humoral xenograft rejection of hDAF transgenic

porcine kidneys in baboons receiving anti-Gal antibody neutralization therapy. *Transplantation.* 2006;81(2):273-283.

23. Amouzegar A, Chauhan SK, Dana R. Alloimmunity and Tolerance in Corneal Transplantation. *J Immunol.* 2016;196(10):3983-3991.

24. Hara H, Cooper DK. The immunology of corneal xenotransplantation: a review of the literature. *Xenotransplantation.* 2010;17(5):338-349.

25. Tan Y, Cruz-Guilloty F, Medina-Mendez CA, et al. Immunological disruption of antiangiogenic signals by recruited allospecific T cells leads to corneal allograft rejection. *J Immunol.* 2012;188(12):5962-5969.

26. Oh JY, Kim MK, Lee HJ, et al. Complement depletion with cobra venom factor delays acute cell-mediated rejection in pig-to-mouse corneal xenotransplantation. *Xenotransplantation.* 2010;17(2):140-146.

27. Cooper DK, Ekser B, Ramsoondar J, et al. The role of genetically engineered pigs in xenotransplantation research. *J Pathol.* 2016;238(2):288-299.

28. Butler JR, Skill NJ, Priestman DL, et al. Silencing the porcine iGb3s gene does not affect Galalpha3Gal levels or measures of anticipated pig-to-human and pig-to-primate acute rejection. *Xenotransplantation.* 2016;23(2):106-116.

29. Bottino R, Wijkstrom M, van der Windt DJ, et al. Pig-to-monkey islet xenotransplantation using multi-transgenic pigs. *Am J Transplant.* 2014;14(10):2275-2287.

30. Dohlman TH, Omoto M, Hua J, et al. VEGF-trap aflibercept significantly improves long-term graft survival in high-risk corneal transplantation.

Transplantation. 2015;99(4):678-686.

31. Kim J, Choi SH, Lee HJ, et al. Comparative efficacy of anti-CD40 antibody-mediated costimulation blockade on long-term survival of full-thickness porcine corneal grafts in nonhuman primates. *Am J Transplant.* 2018;18(9):2330-2341.

32. Choi HJ, Kim MK, Lee HJ, et al. Efficacy of pig-to-rhesus lamellar corneal xenotransplantation. *Invest Ophthalmol Vis Sci.* 2011;52(9):6643-6650.

33. Hara H, Koike N, Long C, et al. Initial in vitro investigation of the human immune response to corneal cells from genetically engineered pigs. *Invest Ophthalmol Vis Sci.* 2011;52(8):5278-5286.

34. Niederkorn JY, Larkin DF. Immune privilege of corneal allografts. *Ocul Immunol Inflamm.* 2010;18(3):162-171.

35. Hori J, Vega JL, Masli S. Review of ocular immune privilege in the year 2010: modifying the immune privilege of the eye. *Ocul Immunol Inflamm.* 2010;18(5):325-333.

36. Jabbehdari S, Rafii AB, Yazdanpanah G, et al. Update on the Management of High-Risk Penetrating Keratoplasty. *Curr Ophthalmol Rep.* 2017;5(1):38-48.

37. Flynn TH, Ohbayashi M, Ikeda Y, et al. Effect of allergic conjunctival inflammation on the allogeneic response to donor cornea. *Invest Ophthalmol Vis Sci.* 2007;48(9):4044-4049.

38. Niederkorn JY. Immune mechanisms of corneal allograft rejection. *Curr Eye Res.* 2007;32(12):1005-1016.

39. Lapp T, Zaher SS, Haas CT, et al. Identification of Therapeutic Targets of

Inflammatory Monocyte Recruitment to Modulate the Allogeneic Injury to Donor Cornea. *Invest Ophthalmol Vis Sci.* 2015;56(12):7250-7259.

40. Funding M, Hansen TK, Gjedsted J, et al. Simultaneous quantification of 17 immune mediators in aqueous humour from patients with corneal rejection. *Acta Ophthalmol Scand.* 2006;84(6):759-765.

41. Maier P, Heizmann U, Bohringer D, et al. Distinct cytokine pattern in aqueous humor during immune reactions following penetrating keratoplasty. *Mol Vis.* 2010;16:53-60.

42. Inomata T, Hua J, Di Zazzo A, et al. Impaired Function of Peripherally Induced Regulatory T Cells in Hosts at High Risk of Graft Rejection. *Sci Rep.* 2016;6:39924.

43. Zhiqiang P, Cun S, Ying J, et al. WZS-pig is a potential donor alternative in corneal xenotransplantation. *Xenotransplantation.* 2007;14(6):603-611.

44. Jie Y, Liu L, Pan Z, et al. Survival of pig-to-rhesus corneal xenografts prolonged by prior donor bone marrow transplantation. *Mol Med Rep.* 2013;7(3):869-874.

45. Choi SH, Yoon CH, Lee HJ, et al. Long-term safety outcome of systemic immunosuppression in pig-to-nonhuman primate corneal xenotransplantation. *Xenotransplantation.* 2018;25(4):e12442.

46. Hosmer DW, Lemeshow S. Applied Logistic Regression, 2nd Ed. New York: Wiley, 2000.

47. Youden WJ. Index for rating diagnostic tests. *Cancer.* 1950;3(1):32-35.

48. Platt JL. New directions for organ transplantation. *Nature.* 1998;392(6679 Suppl):11-17.

49. Armitage WJ, Dick AD, Bourne WM. Predicting endothelial cell loss and long-term corneal graft survival. *Invest Ophthalmol Vis Sci.* 2003;44(8):3326-3331.

50. Larkin DF. Corneal allograft rejection. *Br J Ophthalmol.* 1994;78(8):649-652.

51. Oh JY, Kim MK, Ko JH, et al. Acute cell-mediated rejection in orthotopic pig-to-mouse corneal xenotransplantation. *Xenotransplantation.* 2009;16(2):74-82.

52. Asgari E, Zhou W, Sacks S. Complement in organ transplantation. *Current opinion in organ transplantation.* 2010;15(4):486-491.

53. Choi HJ, Yoon CH, Hyon JY, et al. Protocol for the first clinical trial to investigate safety and efficacy of corneal xenotransplantation in patients with corneal opacity, corneal perforation, or impending corneal perforation. *Xenotransplantation.* 2018:e12446.

54. Koizumi N, Yamasaki K, Kawasaki S, et al. Cytomegalovirus in aqueous humor from an eye with corneal endotheliitis. *Am J Ophthalmol.* 2006;141(3):564-565.

55. Kitazawa K, Sotozono C, Koizumi N, et al. Safety of anterior chamber paracentesis using a 30-gauge needle integrated with a specially designed disposable pipette. *Br J Ophthalmol.* 2017;101(5):548-550.

초 록

목적: 이종각막이식은 동종 공여각막의 대체제로서 연구되어 왔다. 이 연구에서는 α1,3-galactosyltransferase gene 을 knockout 시킨 형질전환 미니돼지 (GTKOm 돼지)-영장류 전층 이종각막이식에서 이식편의 장기 유효성을 분석하고자 하였다. 이 연구에서는 또한 이전의 야생형 SNU 미니돼지-영장류 전층 이종각막이식의 실험 결과를 포함하여 임상적으로 적용이 가능한 이식편 거부 반응을 예측할 수 있는 바이오 마커를 발굴하고자 하였다.

방법: GTKOm 돼지 각막의 장기 유효성 연구를 위해서 총 9 마리 영장류(Chinese rhesus macaques)의 우안에 GTKOm 돼지의 각막을 전층 이식 시행했다. 9 마리의 영장류를 대조군(n = 5)과 CD20 군(n = 4)으로 나눴다. 두 군 모두 전신 tacrolimus, basiliximab, steroid 를 투여했으며, CD20 군은 추가로 항-CD20 항체를 투여했다. 이식편의 부종, 혼탁, 신생혈관형성을 각 0-4 점으로 평가한 뒤 합산하여 이식편 점수(0-12) 를 산정했다. 이식편 점수가 6 점 이상일 경우 이식편의 거부반응으로 진단했다. 작동 및 기억 T 세포, 항 αGal 항체, 항 non-αGal 항체, 공여자 특이 항체, 보체(C3a) 변화를 비교 분석했다. 바이오마커 연구를 위해서는 우안에 돼지 각막을 전층이식 받은

34 마리의 영장류를 분석했다. 이 중 5 마리는 GTKOm 돼지 각막을 이식 받았고, 29 마리는 야생형 SNU 미니돼지 각막을 이식 받았다. 34 마리의 영장류를 거부반응군(전체 또는 늦은)과 생존군 두 그룹으로 분류했다. 이식편이 6 개월 이내에 거부반응을 보인 모든 개체를 전체 거부반응군으로 정의했고, 이식편의 거부반응이 4 주에서 6 개월사이에 발생한 개체를 늦은 거부반응군으로 정의했다. 이식 후 2 주이내에 모든 영장류의 이종 이식편은 거부 반응을 보이지 않았고, 2 주째의 거부반응 예측 바이오마커 분석을 위해서, 전체 거부반응군(n = 16) 또는 늦은 거부반응군(n = 12)을 생존군(n = 18)과 비교했다. 4 주째의 바이오마커 분석에서는 4 주 이내에 거부 반응을 보인 4 마리의 영장류는 제외하였고, 늦은거부반응군(n = 12)을 생존군(n = 18)과 비교했다. 예측 바이오마커 발굴을 위해서 작동 및 기억 T 세포, 항 αGal 항체, 항 non-αGal 항체, 공여자 특이 항체, 보체(C3a) 수치를 분석했다.

결과: GTKOm 돼지 각막의 장기 유효성 연구에서 CD20 군은 이식편의 장기 생존을 보였고(>375, >187, >187, >83 일), 이는 대조군보다(165, 91, 72, 55, 37 일)보다 길었다(P = 0.008). 거부반응이 온 시점에 활성 B 세포는 CD20 군이 대조군보다 낮았다(P = 0.043). 거부반응 시점에 대조군의 방수 C3a 농도가 술 전보다 증가했고(P = 0.043), 비슷한 시기의 CD20 군보다 높았다(P = 0.014). 4 주째와 거부반응이 온 시점의 항-non-αGal IgG 도 대조군에서만 수술 전보다 증가했다(P = 0.013).

예측 바이오 마커 연구에서 2 주째의 $CD8^+IFN\gamma^+$ 세포와 4 주째의 방수 C3a 는 거부반응군에서 유의하게 증가했다. 수신자 조작 특성 곡선 하의 넓이는 4 주째 방수의 C3a 의 값은 0.847, 2 주째 $CD8^+IFN\gamma^+$ 세포의 값은 0.715 이었다. 이는 방수의 C3a 는 우수한, $CD8^+IFN\gamma^+$ 세포는 허용가능한 판별력을 가짐을 의미한다.

결론: 항-CD20 항체를 포함한 면역억제제 조합을 사용하여야 GTKOm 돼지 각막의 전층 이식편의 장기 생존이 가능함을 확인했다. 이는 돼지-영장류 이종각막이식에서 αGal 을 발현하지 않는 돼지 각막을 사용하여도 B 세포와 보체 활성을 억제하는 것이 이식편의 장기 생존에 중요함을 시사한다. 또한 2 주째의 $CD8^+IFN\gamma^+$ 세포와 4 주째 방수의 C3a 는 돼지-영장류 전층 각막 이식에서 거부 반응을 예측하는 신뢰할만한 바이오마커로 향후 이종각막 임상시험에서 거부 반응을 예측하는 기준으로 사용될 수 있을 것이다.

주요어: 항-CD20 항체; 바이오마커; 거부반응; 각막; 이종이식; α1,3-galactosyltransferase gene knockout 형질전환 미니돼지; 영장류

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