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

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Abstract

We investigated the predictive biomarkers for graft rejection in pig-to-non-human primate (NHP) full-thickness corneal xenotransplantation (n = 34). The graft score (0-12) was calculated based on opacity, edema, and vascularization. Scores ≥ 6 were defined as rejection. NHPs were divided into two groups: 1) graft rejection within 6 months; and 2) graft survival until 6 months. In the evaluation of 2-week biomarkers, none of the NHPs showed rejection within 2 weeks and the 34 NHPs were divided into two groups: 1) entire rejection group (n = 16); and 2) survival group (n = 18). In the evaluation of 4-week biomarkers, four NHPs showing rejection within 4 weeks were excluded and the remaining 30 NHPs were divided into two groups: 1) late rejection group (n = 12); and 2) survival group (n = 18). Analysis of biomarker candidates included T/B cell subsets, levels of anti- α Gal IgG/M, donor-specific IgG/M from blood, and C3a from plasma and aqueous humor (AH). CD8⁺IFN γ ⁺ 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 γ ⁺ cells (both the concentration and percentage: 0.715), indicating excellent or acceptable discrimination ability, which suggests that CD8⁺IFN γ ⁺ cells at week 2 and AH C3a at week 4 are reliable biomarkers for predicting rejection in pig-to-NHP corneal xenotransplantation.

Keywords

biomarker; C3a; CD8⁺IFN γ ⁺ cells; cornea; rejection; transplantation; xenotransplantation

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Conflict of Interest Disclosures

The authors declare no competing financial interests.

1. Introduction

Corneal xenotransplantation using pig donors has been investigated as a substitute for human donor corneas.^{1–4} One of the challenges involving xenotransplantation relates to hyperacute rejection mediated by the natural antibody against Gal α 1,3Gal β 1,4GlcNAc-R (α Gal) synthesized by α 1,3-galactosyltransferase because non-human primates (NHPs) and humans carry natural anti- α Gal IgM.⁵ Corneas express α Gal, although at lower levels than vascular endothelial cells do.⁶ Further, the cornea is immune-privileged.^{7–9} Pig-to-NHP corneal xenotransplantation has been reported to result in longer graft survival compared with other solid organ xenotransplantation.¹⁰

Although the success rate of low-risk corneal allotransplantation is greater than 90%,⁸ the rejection rate can be up to 70% in high-risk cases.^{11,12} In allograft rejection, both innate and adaptive immunities are involved.¹³ Th1 cells play an important role.^{14,15} In corneal xenotransplantation, the rejection process may evoke higher innate response in addition to T-cell responses.⁵

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.^{15–18} 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.^{19–21} 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.^{2,3,22}

2. 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).

2.1 Recipient characteristics and study design

Between 2010 and 2018, 38 rhesus macaques which had undergone full-thickness porcine corneal xenotransplantation were included.^{2,3,22} 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 1. Briefly, 29 NHPs grafted with SNU wild-type (WT) miniature pig corneas reported previously,^{2,3,22} and five NHPs grafted with α 1,3-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.^{2,3} NHPs were administered systemic and topical immunosuppressants listed in Table 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: rejection group (entire or late) and survival group (Table 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. In the evaluation of the 2-week biomarkers, none of the NHPs showed rejection and the 34 NHPs were divided into two groups: entire rejection group (n = 16) and survival group (n = 18). In the evaluation of 4-week biomarkers, four NHPs showing rejection within 4 weeks were excluded and the remaining 30 NHPs were divided into two groups: late rejection group (n = 12) and 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.²³

2.2 Graft score and definition of rejection

The corneal graft score (0–12) was calculated based on opacity, edema, and vascularization as described previously.²⁴ Scores ≥ 6 were defined as graft rejection. Success criteria for corneal xenograft are based on 6-month graft survival.²⁴ Therefore, data were analyzed up to 6 months.

2.3 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.^{1,2} 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 μ g/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) (Supplementary Figure S1). Data were presented as the absolute number of cells per unit volume or percentage of peripheral blood mononuclear cells.

2.4 Antibody assay

Plasma anti-αGal IgM/G Abs were measured by ELISA as previously described.²⁵ 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.⁴ Concentrations of DS Abs were semi-quantitatively expressed as mean fluorescence intensity (MFI).

2.5 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.

2.6 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.²³ The value with the maximum Youden index ($J = \text{sensitivity} + \text{specificity} - 1$) was regarded as the optimal cut-off.²⁶ 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.

3. Results

3.1 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 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 (0.94 ± 1.73 and 0.33 ± 1.41 , respectively; $P = 0.145$; Table 4). Both the concentration and percentage of CD8⁺IFNγ⁺ cells at week 2 were significantly higher in the entire rejection group (52.32 ± 51.69 cells/mm³ and $1.13 \pm 1.16\%$, respectively) than in the survival group (17.68 ± 16.26 cells/mm³ and $0.48 \pm 0.56\%$, respectively; all $P = 0.032$), and those at week 4 and last examination showed no group-wise significant differences (Tables 4 and 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- α Gal 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 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 6 and Supplementary Table S1). Excluding GTKO xenografted NHPs, no significant differences in anti- α Gal and DS Abs were found between the rejection and the survival groups (Supplementary Table S2).

3.2 Predictability of presumptive biomarkers for graft rejection within 6 months

The CD8⁺IFN γ ⁺ 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 1).

The AUC of CD8⁺IFN γ ⁺ 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 γ ⁺ cells estimated at 47.15 cells/mm³ (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 Supplementary Table S3.

4. 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.²⁷ As shown in Tables 1 and 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 γ ⁺ 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.²⁸ Corneal edema can be reversed upon early detection of rejection and appropriate management before irreversible graft failure occurs.²⁹ In this regard, the changes in CD8⁺IFN γ ⁺ 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 γ ⁺ 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.^{1,2,4,30}

AH complement activation is related to both innate and adaptive immunity.³¹ 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.¹⁻⁴ 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.³² 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,¹⁻⁴ which is considered as a routine procedure for patients undergoing PCR testing for virus,³³ and can be performed safely with adequate precaution.³⁴

DS IgG and anti- α Gal IgG were not significant predictors of rejection. In WT pig-to-NHPs corneal xenotransplantation, high levels of α Gal epitope or IgG deposits are present in the rejected graft.¹⁻³ Therefore, subgroup analysis was performed to determine whether the inclusion of GTKO porcine corneal grafts in NHPs affected the changes in DS or anti- α Gal Abs as biomarkers (Table 6 and Supplementary Tables S1 and S2). 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 Supplementary Table S2, 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- α Gal 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 γ ⁺ 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. To the best of our knowledge, this study is the first to report predictive biomarkers for graft rejection in corneal xenotransplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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This study was consulted by a statistician at the Medical Research Collaboration Center of our hospital (<http://mrcc.snuh.org>; MRCC No. 2019-0006).

Abbreviations:

Ab	antibody
αGal	Gal α 1,3Gal β 1,4GlcNAc-R
AH	aqueous humor
AU	artificial unit
AUC	areas under the curve
C3a	complement activation fragment
DS	donor pig-specific
GTKO	α -1,3-galactosyltransferase gene knockout
IL	interleukin
IVIG	intravenous immunoglobulin
IFN	interferon
MFI	mean fluorescence intensity
NHP	non-human primate
ROC	receiver operating characteristic
SNU	Seoul National University
WT	wild type

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Figure 1.

Receiver operator characteristic curve analysis of CD8⁺IFN γ ⁺ 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 γ ⁺ cells at week 2 was 0.715 (both concentration and percentage), indicating acceptable discrimination ability. The CD8⁺IFN γ ⁺ cell concentration of 47.15 cells/mm³ (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

Table 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 ²
2	CD154	Anti-CD154 Ab IVIG Methylprednisolone	4	WT	>192, >243, 318, 933	2015 ²
3	CD40	Anti-CD40 Ab IVIG Methylprednisolone	6	WT	41, >196, >203, >273, >422, >511	2018 ³
4	CD20 (Full dose)	Anti-CD20 Ab ^a Tacrolimus ^c IVIG Basiliximab Methylprednisolone	6	WT	134, >184, >210, >260, 297, >470	2018 ³
5	CD20 (Low dose)	Anti-CD20 Ab ^b Tacrolimus ^d IVIG Basiliximab Methylprednisolone	7	WT	56, 92, 162, >181, >182, >182, >198	2018 ²²
6	Tacrolimus only	Tacrolimus ^e IVIG Basiliximab Methylprednisolone	5	GTKO (n = 4), GTKO/hCD 39KI (n = 1)	37, 55, 72, 91, 165	Unpublished
			3	WT	29, 149, 161	2018 ²²

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

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.

Table 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 $\pm$ SD (ranges)	Survival group number	Total number
Week 2	Blood: C3a, DS Abs, anti- α Gal Abs, T and B cell subsets	16 [*]	82.63 $\pm$ 54.38 (21~161)	18	34
Week 4	Blood: C3a, DS Abs, anti- α Gal Abs, T and B cell subsets AH: C3a	12 [†]	101.25 $\pm$ 50.15 (41~161)	18	30

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

^{*} 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.

Table 3.

Baseline levels of biomarker candidates in rejection and survival groups.

Biomarkers	Entire rejection (n = 16)	Late rejection (n = 12)	Survival (n = 18)	<i>P</i> (Entire rejection vs. Survival)	<i>P</i> (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 ⁺ IFNγ ⁺ (cells/mm ³)	12.14 ± 16.74	11.75 ± 17.24	17.02 ± 22.16	0.352 [†]	0.330 [†]
CD8 ⁺ IFNγ ⁺ (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 ⁺ IFNγ ⁺ (%)	0.33 ± 0.47	0.30 ± 0.52	0.51 ± 0.70	0.334 [†]	0.204 [*]
CD8 ⁺ IFNγ ⁺ (%)	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 [†]

* Independent T-test (two-tailed),

† Mann-Whitney U test (two-tailed)

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

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.

Table 4.

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

Biomarkers	Week 2			Last FU		
	Entire rejection (n = 16)	Survival (n = 18)	P	Entire rejection (n = 16)	Survival (n = 18)	P
Graft score	0.94 ± 1.73	0.33 ± 1.41	0.145 [*]	6.56 ± 0.51	0.11 ± 0.47	< 0.001 [*]
AH C3a (ng/mL) ^a	NA	NA	NA	22.01 ± 5.94	6.50 ± 5.69	< 0.001 [*]
Plasma C3a (ng/mL)	167.83 ± 131.33	137.67 ± 62.22	0.863 [*]	183.38 ± 97.42	124.06 ± 52.63	0.028 [*]
DS IgG (MFI)	680.30 ± 804.89	395.23 ± 349.09	0.748 [*]	1124.00 ± 1269.31	240.83 ± 439.75	< 0.001 [*]
DS IgM (MFI)	299.66 ± 534.32	224.14 ± 105.64	0.343 [*]	252.13 ± 278.58	245.18 ± 144.93	0.485 [*]
Anti-αGal IgG (AU/mL)	234.64 ± 248.13	107.31 ± 75.46	0.255 [*]	385.48 ± 677.19	51.99 ± 111.92	0.007 [*]
Anti-αGal IgM (AU/mL)	416.53 ± 337.88	396.11 ± 209.65	0.512 [*]	353.34 ± 220.04	349.73 ± 171.86	0.730 [*]
CD4 ⁺ IFNγ ⁺ (cells/mm ³)	19.41 ± 31.94	7.33 ± 10.52	0.098 [*]	8.19 ± 11.83	15.93 ± 24.74	0.350 [*]
CD8⁺IFNγ⁺ (cells/mm³)	52.32 ± 51.69	17.68 ± 16.26	0.032[*]	33.88 ± 28.99	58.08 ± 52.49	0.142 [*]
CD4 ⁺ CM (cells/mm ³)	397.38 ± 194.67	394.60 ± 377.43	0.317 [*]	352.13 ± 156.95	268.24 ± 125.61	0.093 [†]
CD4 ⁺ EM (cells/mm ³)	40.38 ± 58.57	25.04 ± 32.95	0.558 [*]	31.44 ± 35.26	71.96 ± 107.06	0.617 [*]
CD8 ⁺ CM (cells/mm ³)	133.02 ± 70.14	138.69 ± 130.00	0.605 [*]	138.69 ± 94.06	109.26 ± 56.17	0.270 [†]
CD8 ⁺ EM (cells/mm ³)	307.89 ± 159.73	261.28 ± 253.64	0.121 [*]	498.63 ± 521.24	515.40 ± 430.83	0.641 [*]
ActB (cells/mm ³)	6.40 ± 5.76	3.77 ± 6.02	0.073 [*]	6.88 ± 12.41	4.32 ± 5.68	0.794 [*]
Treg (cells/mm ³)	33.08 ± 32.11	45.21 ± 72.34	0.490 [*]	27.75 ± 18.14	21.80 ± 16.84	0.233 [*]
CD4 ⁺ IFNγ ⁺ (%)	0.48 ± 0.85	0.18 ± 0.25	0.112 [*]	0.32 ± 0.62	0.29 ± 0.33	0.388 [*]
CD8⁺ IFNγ⁺ (%)	1.13 ± 1.16	0.48 ± 0.56	0.032[*]	0.98 ± 1.18	1.41 ± 1.32	0.227 [*]
CD4 ⁺ CM (%)	8.30 ± 2.87	9.69 ± 4.54	0.448 [*]	8.39 ± 2.93	6.80 ± 3.45	0.162 [†]
CD4 ⁺ EM (%)	0.79 ± 1.16	0.66 ± 0.66	0.863 [*]	0.70 ± 0.55	1.29 ± 1.47	0.370 [*]
CD8 ⁺ CM (%)	2.79 ± 1.20	3.26 ± 1.56	0.878 [†]	3.26 ± 1.97	2.77 ± 1.50	0.617 [*]
CD8 ⁺ EM (%)	7.14 ± 4.80	6.35 ± 4.14	0.334 [*]	10.52 ± 6.96	12.51 ± 8.66	0.605 [*]
ActB (%)	0.12 ± 0.10	0.08 ± 0.09	0.138 [*]	0.13 ± 0.22	0.12 ± 0.17	0.822 [*]
Treg (%)	0.81 ± 0.69	0.92 ± 0.97	0.918 [*]	0.80 ± 0.48	0.67 ± 0.54	0.546 [*]

^{*} Mann-Whitney U test (two-tailed),

[†] Independent T-test (two-tailed).

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

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

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.

Table 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 ⁺ IFNγ ⁺ (cells/mm ³)	18.43 ± 29.32	9.03 ± 10.49	0.374 [*]	7.58 ± 12.89	15.93 ± 24.74	0.268 [*]
CD8 ⁺ IFNγ ⁺ (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 ⁺ IFNγ ⁺ (%)	0.45 ± 0.93	0.25 ± 0.26	0.933 [*]	0.27 ± 0.66	0.29 ± 0.33	0.189 [*]
CD8 ⁺ IFNγ ⁺ (%)	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 [*]

* Mann-Whitney U test (two-tailed),

[†]Independent T-test (two-tailed).

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

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.

Table 6.

Summary of subgroup analysis indicating differences in DS IgG and IgM levels in the rejection group according to donor pig type. The DS IgM level 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 GTKO xenografted NHPs without baseline differences, suggesting a possible association between the DS IgG level and rejection in 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)
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
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
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
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

*Mann-Whitney U test (two-tailed)

DS, donor pig-specific; WT, wild type; GTKO, α-1,3-galactosyltransferase gene knockout; NHPs, non-human primates; αGal, galactose-α-1,3-galactose; IFN, interferon
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.