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Long-term safety outcome of systemic immunosuppression in pig-to-nonhuman primate corneal xenotransplantation

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Abstract

Background—Safety concerns exist for corneal recipients under immunosuppression. We report long-term safety results of porcine corneal xenotransplantation under immunosuppression in nonhuman primates.

Methods—Systemic monitoring data from 49 Chinese rhesus macaques that received pig corneal transplant between 2009 and 2018 were retrospectively reviewed. The recipients were divided into 4 groups depending on the systemic immunosuppressants used: (1) conventional steroid group; co-stimulation blockade groups [(2) anti-CD154 antibody, (3) anti-CD40 antibody]; and (4)

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commercially available immunosuppressants (anti-CD20 antibody, tacrolimus, basiliximab) group. We compared results of general condition monitoring; hematologic, biochemical and electrolyte tests; and Rhesus Cytomegalovirus infection monitoring.

Results—All recipients recovered from early weight loss. White blood cell counts significantly decreased at 6 months in the steroid and anti-CD154 groups. Abnormal liver and kidney function and electrolyte imbalance were not observed in all groups. The mean value of Rhesus Cytomegalovirus DNA copies was consistently lower than 200 copies/ml, and antibody titers did not change over time in all groups. Tacrolimus-associated thrombotic microangiopathy was developed in one case, which resolved after discontinuation of tacrolimus. In 2017, a simian varicella virus outbreak led to clinical signs in 5 that received immunosuppressive therapies, of which 3 died.

Conclusion—Co-stimulatory blockade-based and anti-CD20 antibody/tacrolimus-based immunosuppressive therapies seem to be comparably safe with steroid therapy in nonhuman primates receiving corneal xenotransplantation, as they did not reactivate Rhesus Cytomegalovirus and maintained manageable systemic status. Although reactivation is rare, antiviral prophylaxis for simian varicella virus should be considered in immunocompromised hosts.

Keywords

Safety; Cornea; Xenotransplantation; non-human primates; infection; Rhesus Cytomegalovirus; Simian Varicella Virus; Immunosuppression

1 Introduction

Technological advances such as the development of alpha-1,3-galactosyltransferase gene knockout pigs, genome-wide inactivation of porcine endogenous retrovirus (PERV), and the establishment of successful immunosuppression strategies for nonhuman primates (NHP) have increased the feasibility of xenotransplantation clinical trials.^{1–5} The infection risks related to donor-derived pathogens and host-derived opportunistic pathogens under immunosuppression remain a significant challenge to devising clinical trials in xenotransplantation.^{6, 7}

To date, PERV has been a concern as a donor-derived pathogen in xenotransplantation. Importantly, PERV was not transmitted in numerous studies including clinical trials of islet cells, liver cells, and skin xenotransplantation and NHP studies of corneal xenotransplantation; hence, it is uncertain whether PERV poses any risk.^{7–13} Given that the possibility of transmission is independent of the value of the NHP model, the possibility of the transmission remains to be established.^{7, 13} To exclude other potentially transmissible organisms, “designated pathogen free (DPF)” breeding pigs are used to minimize the risk.^{6, 14} Importantly, systemic immunosuppressive therapy may not only increase donor-derived, but also host-derived infections.

We have been investigating the feasibility of corneal xenotransplantation in NHP models since 2009.^{3–5, 15} To minimize the transmissible infection, Seoul National University (SNU) miniature pigs were raised in a DPF environment and maintained with standard operating procedures (SOPs).¹⁶ Donor corneas were prepared using SOPs based on international

guidelines.^{14, 17} Herein, we report long-term outcomes of reactivation of RhCMV disease, adverse reactions, and general welfare in NHPs treated with different immunosuppressants (corticosteroids, co-stimulatory blockade, or anti-CD20 antibody [Ab]/tacrolimus combination) after porcine corneal xenotransplantation.

2 Materials and Methods

2.1 Donors and recipients for keratoplasty

All studies carried out in our laboratory adhered to The Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The primate study protocol was approved by the Research Ethics Committee at Seoul National University Hospital (IACUC No. 09-0156, 11-0152, 12-0207, 13-0221, 15-0171) and Seoul National University (IACUC No. SNU-151102-3).

Corneas were harvested from genetically unmodified, pathogen-free SNU miniature wild type pigs that had been inbred in the DPF (Table S1 and S2) environment of the Xenotransplantation Research Center at Seoul National University Hospital. SNU miniature pigs are also free of zoonotic viruses of concern such as PCV, PCMV and PLHV.¹⁶ Between 2009 and 2018, 49 Chinese rhesus macaques were given pig corneas for penetrating keratoplasty or lamellar keratoplasty. The keratoplasty procedures were previously described.^{3, 4, 15, 18, 19}

2.2 Group analysis depending on immunosuppressants used

Postoperatively, all the recipients received daily topical prednisolone and weekly subconjunctival dexamethasone injection as described in previous studies.^{3, 4, 15, 18, 19} The recipients were divided into 4 groups depending on the systemic immunosuppressants used for maintenance therapy as follows: (1) conventional steroid group as a control (CS, n = 14); co-stimulation blockade group (CoB, n = 15) including two subgroups; (2) CD154 Ab group (CD154, n = 4), (3) Anti-CD40 Ab group (CD40, n = 11)); and (4) commercially available immunosuppressants combination regimen (CA, n = 15) using anti-CD20 Ab (MabThera®, Hoffmann-La Roche, Basel, Switzerland), basiliximab (Simulect®, Novartis Pharmaceuticals Corporation, East Hanover, NJ, USA) and/or tacrolimus (Prograf®, Astellas Pharma US, Deerfield, IL, USA). Administration schedules of each immunosuppressant were previously described (Table 1).^{3, 4, 15, 18, 19}

In CS group, methylprednisolone (Solumedrol; Pfizer, New York, USA) was intramuscularly injected at an initial dose of 2 mg/kg/day and tapered over 5 weeks.

Recombinant anti-CD154 Abs (V-regions from mouse 5C8 clone; C-regions human IgG1k) or anti-CD40 Abs (2C10R4, NIH Nonhuman Primate Reagent Resource) were used in CoB group.²⁰ In the CoB group, in addition to systemic methylprednisolone, intravenous immunoglobulin (IVIG) 1 g/kg was administered on preoperative day 1 and postoperative day 14. The CD154 group additionally received 15 to 19 doses of anti-CD154 Ab intravenously at 20 mg/kg/day; and the CD40 group, 15 doses of anti-CD40 Ab at 30–50 mg/kg/day intravenously.^{3, 4, 19}

In the CA group, 3 different protocols depending on the dose and interval of the anti-CD20 Ab and tacrolimus were executed as follows: (1) Full dosage protocol (n = 7) involved administration of anti-CD20 Ab twice in the first week and thereafter every 8 weeks for 6 months, basiliximab on operation day and POD 4, and tacrolimus twice a day at a dose of 0.05 mg/kg;¹⁹ (2) low dosage protocol (n = 6) involved change of anti-CD20 Ab administration interval from 8 to 12 weeks and reduction in the dosage of tacrolimus to 0.035 mg/kg after 4 weeks of administration at a dose of 0.05 mg/kg; (3) tacrolimus alone protocol (n = 2) involved administration of only tacrolimus twice a day at a dose of 0.05 mg/kg and basiliximab on operation day and POD 4 without administration of anti-CD20 Ab.

We maintained the immunosuppressant protocols mentioned above for 6 months in all the groups. For NHPs that should be followed-up for more than one year, the immunosuppressant protocol was maintained. Tacrolimus and anti-CD20 Ab were only temporally stopped when a subject developed pancytopenia. A simian varicella virus (SVV) infection outbreak occurred in the facility when experimentation with lower dosage or tacrolimus only protocols (CA) was underway. This resulted in discontinuation of tacrolimus in infected NHPs during the outbreak. Therefore, we excluded 5 SVV-infected primates from the group analysis mentioned above and reported the incident separately.

2.3 Graft survival

Graft clarity, edema, and neovascularization were evaluated by slit-lamp biomicroscopy. Rejection score was graded based on previous study and international consensus.^{14, 15}

2.4 Clinical safety evaluations

2.4.1 General condition monitoring—NHPs were closely monitored daily, including measurement of body temperature and weight, and any unusual symptoms and signs were immediately reported.

2.4.2 Hematological and biochemical tests—In all transplanted groups, complete blood count was analyzed every 2 weeks until 6 months, using an automatic hematological analyzer (ADVIA2120i; SIEMENS Healthcare Diagnostics Inc., Tarrytown, NY, USA). Serum biochemistry and electrolytes were measured using a serum chemistry analyzer (HITACHI7180; Hitachi High-Technologies Corporation, Tokyo, Japan) and evaluated as previously reported.²¹ Biochemical and electrolyte tests were performed at 2-week intervals until 1 month in the CS group and 6 months in the other groups.

2.4.3 Quantitation of Rhesus Cytomegalovirus (RhCMV)—The quantification of RhCMV DNA in plasma was determined by quantitative real time polymerase chain reaction (PCR) using forward and reverse primers, 5' – ACAGAGGCCAGTGGGATGTC – 3' and 5' – CCCTGATGATGGGCATAGATAAG – 3', and a probe, 5' FAM – CCAGGCACATTCTCTGGGAGCACAC-3' TAMRA (Bioneer, Daejeon, South Korea). Their sequences are located in the highly conserved RhCMV 156 region and are specific to RhCMV. The PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used for the quantification of the signals.²²

2.4.4 Anti-RhCMV Ab measurement—Anti-RhCMV Ab titer was measured by indirect immunofluorescent staining.²³ Serum was diluted twofold serially from 1:10 for immunoglobulin G (IgG) and incubated with acetone-fixed RhCMV 68-1 (ATCC VR-677)-infected HEL299 for 30 minutes at room temperature. After washing, fluorescein isothiocyanate-conjugated anti-monkey IgG Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USG) was added. Fluorescence was observed under an Axioscope fluorescent microscope (Zeiss, Germany). The Ab titer was defined as the highest serum dilution, which shows bright fluorescence.

2.4.5 Outbreak of SVV infection and death/infection rate analysis in the presence of immunosuppressants—Outbreak of SVV infection occurred in the primate facility from March 16, 2017 to April 27, 2017. Genomic DNA was extracted from whole blood for real time PCR. Real time PCR complementary to SVV open reading frame (ORF) 21 was conducted in Bionics Inc. (Seoul, Korea) using the primers as follows: forward primer 5' –AGACAGGGAATGGTAGAAAGTG–3', reverse primer 5' –ACTTGACACGCTGTGTTAGA–3'. Serological antibody assay for SVV was conducted in the VRL laboratory (Suzhou, CHINA) using ELISA. Clinically relevant infection was diagnosed based on the symptoms with erythematous vesicular rash, loss of appetite, fever, or increase of aspartate aminotransferase (AST) / alanine transaminase (ALT) level. Intravenous ganciclovir (Cymevene®, Hoffmann-La Roche, Basel, Switzerland) 5 mg/kg was given for infected NHPs and prophylactic oral valganciclovir (Valcyte®, Hoffmann-La Roche, Basel, Switzerland) 15 mg/kg was used for uninfected NHPs.

During the outbreak, 5 out of 11 primates in the CA protocol group showed clinical symptoms of SVV-infection. To compare the infection and death rates between the immunocompetent and immunocompromised hosts during the outbreak, we collected whole NHPs data with/without corneal transplantation in the facility (n = 48) between March 16, 2017 and April 27, 2017. In addition to the 11 NHPs that received corneal transplants, 37 other NHPs were also housed in this facility. Eleven of them were receiving immunosuppressants after transplants of organs other than the cornea, while the remaining 26 did not receive immunosuppressants as they had not received transplants yet.

2.5 Statistical analyses

GraphPad Software (GraphPad Prism, Inc., La Jolla, CA, USA) was used. Mean values of parameters were compared between the groups preoperatively and after 1 and 6 months using the Mann-Whitney U test. The time-dependent changes in parameters were compared between the preoperative state and 1 and 6 months using the Wilcoxon signed rank test. Data are presented as mean ± standard error (SE). For the analysis of two-by-two tables (infection and death rate), Chi-square or Fisher-exact test was used. Statistical significance was accepted for p values < 0.05.

3 Results

3.1 General condition monitoring in group analysis

All the 44 NHPs maintained good condition, appetite, and physical activity during the study period except for the early weight loss as follows; All groups except the CS group showed a tendency to lose weight at 1 month. The CD40 group in particular, showed a significant decrease in BW at 1 month ($p = 0.0225$, Wilcoxon signed rank test) but recovered at 3 and 6 months (Figure 1A). The CD154 group showed a 10% weight loss at 3 months after surgery despite good dietary intakes and good physical activities but recovered after supportive care. The weight change was not statistically significant due to the small number. Body temperature remained stable except for the decrease at 1 month in CD40 group ($p = 0.0140$, Wilcoxon signed rank test), but remained within the normal ranges reported in previous reports (Figure 1B).²⁴

3.2 Hematological tests

White blood cell (WBC) counts were reduced at 6 months in the CS ($p = 0.0156$, Wilcoxon signed rank test) and CD154 ($p = 0.0190$, Wilcoxon signed rank test) groups compared with the preoperative level but remained constant throughout the study period in the CD40 and CA groups (Figure 2A). Hb level tended to decrease in the CD40 ($p = 0.0078$) and CA ($p = 0.0022$, Wilcoxon signed rank test) groups at 1 month; however, the Hb level recovered at 6 months (Figure 2B).

3.3 Biochemical tests

AST and ALT levels remained stable over time without significant changes in all groups (Figure 3A, B). There was no difference in blood urea nitrogen (BUN) level between groups postoperatively. In the CA group, the BUN level temporarily increased at 3 months ($p = 0.0170$, Wilcoxon signed rank test) (Figure 3C). Creatinine decreased steadily in the CD40 group (pre-op vs 6 months; $p = 0.0391$, Wilcoxon signed rank test) and temporally dropped in the CA group (pre-op vs 1 months; $p = 0.0063$, Wilcoxon signed rank test), but the changes were within normal ranges (Figure 3D). In all groups, blood glucose level did not change with time (Figure 3E).

3.4 Electrolyte tests

There was no difference in mean value for sodium (Na) between the groups. The value for Na decreased at 6 months in CA group ($p = 0.0201$, Wilcoxon signed rank test) but remained within the normal range (Figure 4A). The potassium (K) values of the CA and CD40 group tend to increase outside normal ranges, which did not show any statistically significant changes with time in all groups (Figure 4B).

3.5 RhCMV monitoring

Prior to xenotransplantation, all NHPs in the CD154 and CA groups did not show RhCMV DNA copies, while some of the NHPs in the CD40 group showed low levels of RhCMV viremia (< 50 copies /ml) or CS groups (< 200 copies /ml). The mean value of RhCMV DNA copies was lower than 200 copies/ml in all groups and did not increase over the 6 months of

treatment (Figure 5A). The preoperative RhCMV Ab titer did not elevate with time in all groups (Figure 5B).

3.6 Outbreak of the simian-varicella virus and death/infection rate analysis in the presence of immunosuppressants

During the outbreak, 11 out of 48 NHPs housed in the facility (22.4%) showed similar clinical symptoms, and 4 of them died (8.2%). SVV PCR and ELISA were positive in 24.4% and 44.4% of the primates. Among the 11 clinically infected NHPs, 6 were under immunosuppression therapy (27.3%) while 5 were immunocompetent (19.1%). Infection rates were not different between immunosuppressed and immunocompetent NHPs (Table 2, $p > 0.05$). However, there was a non-significant trend of a higher death rate among immunosuppressed hosts (66.7% vs 0%; $p > 0.05$, Fisher-exact test) (Table 2). Of the 11 NHPs in the CA group (anti-CD20/tacrolimus) with corneal transplantation, 5 had clinical symptoms and 3 of them died. No additional SVV infections occurred from April 27, 2017, when we started administering intravenous ganciclovir to infected NHPs and prophylactic valganciclovir to uninfected NHPs.

3.7 Other systemic complications of immunosuppression excluding SVV

No other systemic complications were found in the CS or CoB group. In the CA group, systemic infection occurred in 3 cases (shigellosis in 2 and pneumonia in 1) and was successfully treated with antibiotic therapy (Table 3, no statistical difference between the groups).

Other than infections, 1 NHP in CA group showed tacrolimus-associated thrombotic microangiopathy. Increased levels of lactate dehydrogenase, bilirubin, and decreased levels of Hb and platelets recovered after cessation of tacrolimus and administration of dalteparin and clopidogrel. These hematologic abnormalities recurred when tacrolimus was re-administered, confirming the diagnosis. The laboratory tests normalized after discontinuation of tacrolimus and re-administration of dalteparin.²⁵

4 Discussion

Corticosteroid therapy alone is sufficient for xenotransplantation using decellularized cornea, but co-stimulation blockade or combination of anti-CD20 Ab and tacrolimus is more successful for LKP or PKP using cellular cornea.^{3, 4, 15, 18, 19} Our study indicates that in corneal xenotransplantation, co-stimulation blockade-based or anti-CD20 Ab- and tacrolimus-based immunosuppression is not only effective for graft survival, but also safely tolerated for 6 months in NHPs compared to steroids. One-year systemic monitoring results in 9 NHPs under immunosuppression also showed no statistically significant changes in data of 1 year compared with preoperative level or 6 months (Supplementary Figure S1).

Transient weight loss in the CD40 group presumably occurred as a result of physical difficulty with feeding that the NHPs faced due to neck collars applied to prevent them from touching their eyes for the first 2 weeks. After removing the neck collars, the NHPs regained their normal weight. Although not significantly, two NHPs in the CD154 group also showed a weight loss of more than 10% at 3 months. Neither decreased dietary intake nor abnormal

laboratory findings were observed. We were advised close monitoring of the NHPs by veterinary physicians. Thereafter, the NHPs gradually recovered body weight without any further problems. Since all the studies were carried out sequentially and not simultaneously, different environment may have affected group-to-group variations. However, the results did not show clinically significant difference between the groups, as most laboratory test values were within the normal range provided by a previous report.²¹ Decrease in WBC counts in the CS and CD154 groups suggests that bone marrow suppression should be carefully monitored in this protocol. Given the wide normal ranges in NHPs,²¹ and variable preoperative status in each group, liver and kidney function were tolerably maintained over 6 months in all groups. Although it is non-significant, the potassium (K) values should be also carefully monitored in NHPs using CA and CD40 protocol.

There are two aspects of infectious risks in corneal xenotransplantation: (1) The transmission of infection from the donor pigs' corneal cells and (2) a host-derived reactivation of the latent virus or newly developed opportunistic infection in an immunocompromised host due to immunosuppression. For the main issue of transmissible infection, PERV, our group has previously reported no observed PERV transmissions.¹¹ We investigated the possibility of reactivation of the RhCMV virus throughout the study because viruses such as CMV, varicella-zoster virus (VZV), and herpes simplex virus are known to be reactivated by the use of immunosuppressants.^{26, 27} It is clinically translatable because RhCMV is well established as a model for human CMV (HCMV) and the Ab measurement method used is also applicable to humans.²³ Rhesus macaques are a widely used experimental animal species and, RhCMV contains most of the HCMV gene families, thus allowing the study of their role in CMV infection.²⁸ In our study, RhCMV copies were detected in many NHPs but were much lower than the viral load of 1,000–100,000 copies/ml that is detected in active CMV disease.²⁹ Additionally, no clinically symptomatic RhCMV disease was observed, which may be related to the fact that all our NHPs had RhCMV Abs before transplantation. Given that there is no RhCMV disease in the protocol (anti-CD20/tacrolimus) of our study, this immunosuppressive regimen can be considered as tolerable when compared to the facts that 1) 5.56 %–41.4% of CMV disease rates in kidney allotransplant recipients under a similar triple immunosuppressant regimen,^{30, 31} and 2) there is currently no searchable report about reactivation of CMV disease in corneal allotransplant recipients.^{32–34} In high-risk corneal allotransplantation, systemic cyclosporine, tacrolimus, or mycophenolate mofetil can be administered systemically.^{35, 36} As far as the authors know, there has been no report of serious pathogen reactivation under immunosuppression from these studies on corneal allotransplantation presumably because most of the studies used one drug (mono-therapy).^{32–34, 36, 37} Considering that high dosage of triple combination of immunosuppressants (anti-CD20 antibody, tacrolimus, basiliximab) is used in corneal xenotransplantation, it would be reasonable to evaluate whether the effect of our triple immunosuppressants regimen on the CMV disease in xenotransplantation is comparable to effect of the other triple immunosuppressants regimen (anti-thymocyte globulin, tacrolimus with either mycophenolate or azathioprine)³⁰ or low dosage of the similar triple immunosuppressants regimen (200–500 mg of anti-CD20 antibody, either tacrolimus or cyclosporine, basiliximab)³¹ in clinical allotransplantation such as kidney transplantation. The frequency of CMV disease occurrence is 41.4% with the former

regimen and 5.56% with the latter regimen.^{30, 31} Therefore, with regard to CMV reactivation, our work is novel in that it reveals that the administration of high dosage of anti-CD20 Ab combined with tacrolimus/basiliximab for xenotransplantation may be comparably safe with the regimens that have been conventionally used in clinical settings for allotransplantation. Interestingly, low level of RhCMV viremia spontaneously resolved in the CS group. The spontaneous resolution of low level of CMV viremia (<200 copies/ml) has also been reported in patients with transplantation.³⁸ There is a need to investigate whether steroids have a role in the resolution of RhCMV infection.

Simian varicella virus (SVV) is closely related to VZV and induces a natural varicella-like disease in nonhuman primates. Disseminated SVV infection in immunocompromised NHPs was associated with a high fatality rate during the outbreak (66.7%). Since this was the first SVV outbreak at our primate center, the diagnosis of the disease was delayed and the first 3 infected NHPs did not receive adequate antiviral treatment, leading to death. Disseminated VZV infection is very rare with a high mortality rate in humans. According to one retrospective study, only 56 renal transplant recipients had a disseminated VZV infection during 26 years, and the mortality rate was 30%.²⁷ From this event, we learned that antiviral prophylaxis is important even for rare diseases, because of the high fatality rate in immunocompromised conditions. According to our study, other infections associated with immunosuppression seem to be tolerable if they are detected early through close monitoring.

As for bacterial or protozoal infections, we reported a rate of 6.8% among all NHPs under immune suppression therapy throughout the whole trial. However, the infection rate went up to 25% in NHPs under anti-CD 20 Ab/tacrolimus regimen, although the difference was not statistically significant. Our triple immunosuppressant regimen (anti-CD 20 Ab/tacrolimus/basiliximab) showed comparable safety with that described in a previous report, which presented a 27.3% bacterial or protozoal infection rate in patients who received anti-CD20 Ab.³⁹

An important limitation of this study concerns missing data in the CS or anti-CD 154 groups as we conducted experiments using CS or anti-CD154 Ab at initial period (2009–2012) when the protocol had not been fully set-up for the safety monitoring. Given the role of CS group as control and that we can compare the post-immunosuppression changes of each group to the pre-immunosuppression level (control), our study is still meaningful despite some missing data. The following are additional limitations of our study: (1) all the studies were carried out sequentially and not simultaneously (the CS group was performed from 2009 to 2012, the CoB group was performed from 2011 to 2016, and the CA group experiment was performed from 2015 to the present) The environment in each group may not be exactly the same and may affect the preoperative status. (2) this is not a complete pathogen safety study or an outbreak study.

In conclusion, our study is noteworthy in that we report the long-term safety-related issues for systemic immunosuppression in corneal xenotransplantation. It draws attention to the need for proper monitoring and prophylaxis to reduce infection and immunosuppressant-related adverse reactions in future clinical trials for corneal xenotransplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Ab	Antibody
CMV	Cytomegalovirus
RhCMV	Rhesus Cytomegalovirus
DPF	Designated pathogen free
αGal	Galalpha1,3Galbeta1,4GlcNAc-R
LKP	Lamellar keratoplasty
NHP	Nonhuman primate
PKP	Penetrating keratoplasty
PBMC	Peripheral blood mononuclear cell
PERV	Porcine endogenous retrovirus
SOPs	Standard operating procedures
BW	Body weight
POD	Postoperative day
Preop	Pre-operation
SVV	Simian-varicella virus
Na	Sodium
K	Potassium
IVIG	Intravenous immunoglobulin

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Figure 1. Body weight and body temperature change monitoring

(A) The postoperative/preoperative body weight ratios of the conventional steroid group (CS), anti-CD154 Ab group (CD154), anti-CD40 Ab group (CD40), and commercially available immunosuppressants combination regimen group (CA) were calculated at 1 month, 3 months, and 6 months postoperatively. (B) Body temperature of the CD40 and CA group preoperatively and 1 month, 3 months, and 6 months postoperatively.

Figure 2. Hematological tests

(A) Changes in WBC counts of the conventional steroid group (CS), anti-CD154 Ab group (CD154), anti-CD40 Ab group (CD40), and commercially available immunosuppressants combination regimen group (CA) before transplantation and at 1, 3, and 6 months after transplantation. (B) The changes in Hb levels observed before surgery and at 1, 3, and 6 months after transplantation.

Figure 3. Biochemical tests

(A, B) AST and ALT measured preoperatively and at 1, 3, and 6 months postoperatively in conventional steroid group (CS), anti-CD154 Ab group (CD154), anti-CD40 Ab group (CD40), and commercially available immunosuppressants combination regimen group (CA). (C) BUN values before the transplantation and 1, 3, and 6 months after transplantation collected in CS, CD154, CD40, CA groups. (D) Mean values for creatinine in CS, CD154, CD40 and CA groups evaluated preoperatively and 1, 3, and 6 months postoperatively. (E) Serum glucose level measured preoperatively and at 1, 3, and 6 months postoperatively in CS, CD154, CD40, and CA groups.

Figure 4. Electrolyte tests

(A) The values for Na were monitored before surgery and at 1, 3, and 6 months after the surgery in the conventional steroid group (CS), anti-CD154 Ab group (CD154), anti-CD40 Ab group (CD40), and commercially available immunosuppressants combination regimen group (CA). (B) The K values of the CS, CD154, CD40, and CA groups were measured preoperatively and at 1, 3, and 6 months postoperatively.

Figure 5. RhCMV monitoring

(A) The copy number of RhCMV DNA was measured in the conventional steroid group (CS), anti-CD154 Ab group (CD154), anti-CD40 Ab group (CD40), and commercially available immunosuppressants combination regimen group (CA) before transplantation and at 1, 3, and 6 months after the transplantation. (B) The RhCMV Ab titer was also measured preoperatively and at 1, 3, and 6 months postoperatively in the CS, CD154, CD40, and CA groups.

Table 1

Immunosuppressive regimen and graft survival of the non-human primates.

Group [†]	Immunosuppressive regimen	Surgical procedure	Subject number	Graft survival (day)	Mean follow-up (day)
CS	Methylprednisolone [‡]	LKP	4	69, 112, >194, >398	213.0 ± 133.1
		PKP	5	7, 21, 28, 29, 42	29.4 ± 15.0
		Decell-PKP	5	49, >195, >208, >265, >391	261.8 ± 76.8
CD154	Methylprednisolone IVIG [§] Anti-CD154 Ab [¶]	PKP	4	>192, >243, 318, 933	468.3 ± 386.2
CD40	Methylprednisolone IVIG Anti-CD40 Ab	LKP	5	>61 [‡] , >201, >236, >382, >389	253.8 ± 136.9
		PKP	6	41, >196, >203, >273, >422, >511	283.2 ± 155.6
CA (Full Dose)	Methylprednisolone IVIG Anti-CD20 Ab [*] Tacrolimus [#] Basiliximab	PKP	7	>97 [‡] , 134, >184, >210, >260, 297, >470	251.4 ± 116.8
		PKP	6	56, 92, >158, 162, >181, >198	166.8 ± 22.9
CA (Tacrolimus Alone)	Methylprednisolone IVIG Tacrolimus [#] Basiliximab	PKP	2	29, 149	120.5 ± 48.8

CS: conventional steroid group, Ab: antibody, CD154: anti-CD154 Ab group, CD40: anti-CD40 Ab group, CA: commercially available immunosuppressants group, IVIG: intravenous immunoglobulin, LKP: lamellar keratoplasty, PKP: penetrating keratoplasty, Decell: decellularized cornea

[†] Five simian-varicella virus (SVV)-infected primates of CA (low dose or tacrolimus alone) groups were excluded from the group analysis.

[‡] Methylprednisolone was intramuscularly injected at an initial dose of 2 mg/kg/day and tapered over 5 weeks.

[§] IVIG was administered on preoperative day 1 and postoperative day 14 at a dose of 1 g/kg.

[¶] Anti-CD154 Ab was injected intravenously 15 to 19 times at a dose of 20 mg/kg.

^{*} Anti-CD40 Ab was injected intravenously 15 times at a dose of 30–50 mg/kg.

[#] Anti-CD20 Ab was injected intravenously at a dose of 20 mg/kg on postoperative day 0, 7, 63, 119, 175

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Anti-CD20 Ab was injected intravenously at a dose of 20 mg/kg on postoperative day 0, 7, 91, 175

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Tacrolimus was administered intramuscularly twice a day at a dose of 0.05 mg/kg.

Tacrolimus was administered intramuscularly twice a day at a dose of 0.05 mg/kg for 4 weeks followed by 0.035 mg/kg. To prevent further viral reactivation after SVV outbreak, tacrolimus was stopped in all subjects from April 27, 2017 to June 21, 2017.

Basiliximab was administered intravenously at a dose of 0.3 mg/kg on postoperative day 0 and 4.

^d Accidentally died due to asphyxia after the sedation.

^h Died of sepsis due to bowel perforation during blood sampling.

Table 2
Infection and death rate in disseminated simian varicella virus infection under immunosuppression during the outbreak from March to April of 2017

A. Infection and death rate was not different with or without immunosuppression in the total non-human primate population (n = 48) of our facility

Infection rate	IS (+)	IS (-)
Rash and systemic Sx (+) [§]	6 [‡] (27.3%)	5 (19.2%)
Rash and systemic Sx (-)	16	21
p > 0.05, Chi-square test		
Death rate in infected NHPs	IS (+)	IS (-)
No death	2	5
Death	4 [‡] (66.7%)	0 (0%)
p > 0.05, Fisher-exact test		

[‡]5 NHPs with corneal transplants were included.

[‡]3 NHPs with corneal transplants were included.

[§]Systemic symptom include loss of appetite, fever, or increase in aspartate aminotransferase (AST) / alanine transaminase (ALT) level

IS indicates Immunosuppression

Table 3

Systemic infection with the exception of simian varicella virus.

Infection rate	CS	CoB	CD20
Infection(-)	14	15	12
Infection(+)	0	0	3 [†]

[†] 1 case of pneumonia and 2 cases of shigellosis, which were controlled with antibiotic treatment.