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의학박사 학위논문

Comparison of efficacy and safety of anti-CD40 antibody-mediated co-stimulation blockade and anti-CD20 antibody and tacrolimus based combination regimen in long-term survival of full-thickness porcine corneal grafts in nonhuman primates

돼지-영장류 간 이종 전층각막이식에서 항 CD40 단일클론항체를 이용한 면역억제요법과 항 CD20 단일클론항체 및 타크로리무스를 포함한 복합면역억제요법의 장기 유효성과 안전성의 비교 분석

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김 재 영

Comparison of efficacy and safety of anti-CD40 antibody-mediated co-stimulation blockade and anti-CD20 antibody and tacrolimus based combination regimen in long-term survival of full-thickness porcine corneal grafts in nonhuman primates

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Comparison of efficacy and safety of anti-CD40 antibody-mediated co-stimulation blockade and anti-CD20 antibody and tacrolimus based combination regimen in long-term survival of full-thickness porcine corneal grafts in nonhuman primates

by

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A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Ophthalmology at Seoul National University College of Medicine

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Abstract

Comparison of efficacy and safety of anti-CD40 antibody-mediated co-stimulation blockade and anti-CD20 antibody and tacrolimus based combination regimen in long-term survival of full-thickness porcine corneal grafts in nonhuman primates

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Purpose: Many countries are suffering from shortage of donor corneas, and eligible donor pool will decrease. Porcine corneas will be good substitutes for human corneas, however antigenic difference is one of the most serious challenges. We aimed to compare the efficacy and safety of anti-CD40 antibody with those of rituximab-based regimen on the survival of full-thickness corneal grafts in pig-to-rhesus xenotransplantation. In addition, our ultimate goal was to choose a more effective and safer immunosuppressant protocol for clinical trials.

Methods: Thirteen Chinese rhesus macaques consecutively underwent full-thickness corneal transplantation using porcine corneas. Six primates were

administered anti-CD40 antibody (2C10R4), and others were administered rituximab, basiliximab, and tacrolimus. Graft survival, changes in effector and memory T and B cell subsets, donor-specific and anti-αGal antibodies, and aqueous complement were evaluated. Systemic adverse reactions were monitored.

Results: Both anti-CD40 antibody (>511, >422, >273, >203, >196, 41 days) and anti-CD20 antibody (>470, 297, >260, >210, >158, 134, >97days) achieved long-term survival. In the anti-CD20 group, the number of activated B cells was significantly lower than that in the anti-CD 40 group (p=0.0216, Mann-Whitney test), and the level of aqueous complements at 6 month was significantly higher than preoperative level (p=0.0085, Friedman test). There was no difference in the levels of T cells, donor-specific and anti-αGal antibodies between two groups. In the anti-CD20 group, although three primates suffered from adverse reactions, all of them were manageable.

Conclusion: Both anti-CD40 antibody and anti-CD20 antibody protocol are effective on the long-term survival of full-thickness corneal xenotransplantation with less adverse reactions in anti-CD40 treatment. Therefore, it is necessary to adjust the dose of anti-CD20 antibody protocol to reduce side effects in order to use this clinically available regimen immediately in further studies.

Keywords: anti-CD40 antibody, anti-CD20 antibody, basiliximab, tacrolimus, cornea, penetrating keratoplasty, nonhuman primates, xenotransplantation

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Introduction

Corneal transplantation is the treatment of choice for corneal blindness.[1] Shortage of donor corneas and organ trafficking in developing countries are one of the important reasons to explore alternative treatments, including xenotransplantation.[2-4]

It was reported that one of the major obstacles for xenotransplantation is a hyperacute rejection resulted from the specific interaction between the human natural antibody (Ab), termed anti-Gal Ab, and a carbohydrate epitope, termed α-galactosyl epitope (αGal), abundantly expressed on mammalian cells of nonprimate grafts.[5]

In a previous study, gradual increase of plasma IgG anti-Gal Ab levels was found in fresh porcine cornea-grafted α-1,3-galactosyltransferase gene knockout (GTKO) mice, and it is suggested that Ab-mediated rejection through αGal responses may affect the long-term survival of porcine corneal xenograft.[6] In addition, Oh et al. reported that it was T cells rather than NK cells that played a critical role in acute rejection after pig-to-mouse corneal xenotransplantation,[7] and showed that the temporary complement depletion significantly reduced the infiltration of both $CD4^+$ T cells and macrophages within the grafts and delayed an acute cell-mediated rejection in pig-to-mouse corneal xenotransplantation.[8] Therefore, we focused the immunosuppressants targeting B and T cells, and complements, and designed further studies.

Current data on the clinical efficacies of pig-to-nonhuman primate (NHP) xenotransplantation present encouraging outcomes in lamellar keratoplasty (LKP) or

penetrating keratoplasty (PKP) using CD154-CD40 co-stimulatory pathway blockade.[9] CD154, the natural ligand for CD40, is expressed predominantly on activated $CD4^+$ T cells, and has also been described on other cells such as basophils, eosinophils, platelets, activated B cells, and monocytes.[10-12] CD40 is expressed primarily on antigen-presenting cells (APCs) such as dendritic cells, B cells and monocytes.[10,11] Because interactions between CD154 and CD40 are essential for the generation of both T cell-dependent, humoral immune responses and cytotoxic T-lymphocyte responses,[12] the full-thickness corneal grafts of wild-type (WT) Seoul National University (SNU) miniature pigs showed long-term survival in NHPs with anti-CD154 Ab-mediated blockade.[13] Despite these successful results, the immunosuppressive regimen could not be applied to clinical study, because it was reported that the anti-CD154 Abs had serious adverse events such as thromboembolic complications.[14] Therefore, we applied the antagonistic anti-CD40 Ab instead of anti-CD154 Ab and tried to performed LKP first,[15] because anti-CD40 Ab might have lower potency than anti-CD154 Ab and the rejection rate of LKP is lower than that of PKP in allotransplantation.[1] In the previous study of pig-to-primate LKP, long-term survival of partial-thickness porcine corneal grafts was achieved without any systemic adverse events.[15] Based on this encouraging data, in this study, we aimed to apply the same protocol to full-thickness porcine corneal graft transplantation in NHP, because PKP is widely used as the first-line procedure.[1] In addition, we investigated another immunosuppressive regimen including only clinically available medicines, because our goal is to develop a less toxic but effective immunosuppressant protocol in PKP for clinical trials. We hypothesized that an anti-CD40 Ab-based regimen or a commercially available regimen (anti-

CD20 Ab/basiliximab/tacrolimus) may be effective. We compared the efficacy and safety of an anti-CD40 Ab with those of an anti-CD20 Ab and tacrolimus based regimen in pig-to-rhesus full-thickness corneal transplantation to choose a more effective and safer immunosuppressant protocol for clinical trials.

Materials and Methods

All procedures used in this study conformed to the ARVO Statement Regarding the Use of Animals in Ophthalmic and Vision Research. The primate study protocol was approved by the Research Ethics Committee at SNU Hospital [IACUC No. 15-0171-S1A1]. The following methods to evaluate efficacy and safety are basically the same as the methods used in our previous studies.[13,15]

Donors and recipients

Porcine corneas were obtained from pathogen-free SNU miniature WT pigs that had been inbred in the Xenotransplantation Research Center at SNU (Table 1). Thirteen Chinese rhesus macaques were used as recipients (Table 2). The NHPs were divided into two groups based on immunosuppressive regimen; (1) the anti-CD40 Ab-treated group (anti-CD40, n=6) and (2) the combination regimen (anti-CD20 Ab/basiliximab/tacrolimus)-treated group (anti-CD20, n=7).

Orthotopic corneal transplantation

All PKPs with sizes of 7.5/7.0 mm (donor/recipient) were performed by one surgeon, MK Kim, using the same technique described in a previous study.[13]

Postoperative immunosuppressive regimen

All recipients received the same basic medications as follows[15]; 0.5% Levofloxacin and 1% prednisolone acetate were topically administered once per day for 3 months.

Dexamethasone at 1.5 mg/0.3 ml was subconjunctivally injected weekly for 6 months, and methylprednisolone was intramuscularly injected at an initial dose of 2 mg/kg/day, which was tapered over 5 weeks. Intravenous immunoglobulin G (IVIG) was administered at a dose of 1 g/kg on day 0 and after 2 weeks. Aflibercept (VEGF-trap; Eylea® , Regeneron, Tarrytown, NY and Bayer HealthCare, Berlin, Germany) was subconjunctivally injected on day 0.

In the anti-CD40 group, a mouse-rhesus chimeric monoclonal anti-CD40 Ab (2C10R4, NIH Nonhuman Primate Reagent Resource)[16] was administered intravenously at a dosage of 50-30 mg/kg/day on day -1; on postoperative days (PODs) 1, 4, 7, 10, and 14; then once weekly until week 4; every 2 weeks thereafter until week 12; and then every 4 weeks until week 24 (15 doses in total, 50 mg/kg/day for the first 8 doses, and 30 mg/kg/day for the remaining 7 doses).[15]

In the anti-CD20 group, an anti-CD20 Ab (MabThera® , Hoffmann-La Roche, Basel, Switzerland) and basiliximab (Simulect® , Novartis Pharmaceuticals Corporation, East Hanover, NJ) were administered twice in the first week. Anti-CD20 Ab was administered every two months until 6 months. Tacrolimus (Prograf® , Astellas Pharma US, Deerfield, IL) was administered two times per day at a dose of 0.05 mg/kg in most primates. In a recipient (anti-CD20 #6), administration of tacrolimus was temporarily stopped at POD 70 because the primate had pancytopenic laboratory findings. Therefore, we reduced the dose to 0.035 mg/kg after 5 weeks in the last recipient (anti-CD20 #7) to reduce tacrolimus-related complications.

Clinical evaluations

Grading of rejection was performed by evaluating the opacity of the graft, the presence of edema, the vascularization of the graft, central corneal thickness (CCT), intraocular pressure (IOP), and density of endothelial cells (n=10).[13,15] The Kaplan-Meier survival curves were evaluated in both groups.

Histology

Hematoxylin and eosin (H&E) and immunohistochemical/immunofluorescence staining were performed as previously described.[13,15] The primary Abs used are as follows: anti-CD68 Ab (Thermo Scientific, Runcorn, United Kingdom; 1:100), anti-CD3 Ab (1:200; Abcam, Cambridge, MA), anti-CD8 Ab (1:150; Abcam), anti-CD4-alexa Fluor 488 conjugated Ab (1:50; Novusbio, Littleton, CO), and anti-CD20 Ab (1:100; eBioscience, San Diego, CA). 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.

Memory T cell and B cell assays

Changes in memory T cell populations ($CD28^{+}CD95^{+}$ central and $CD28^{-}CD95^{+}$ effector memory cells) and B cells were evaluated in whole blood and draining lymph nodes after the animals were sacrificed.[13,15] In the subjects with surviving grafts, cell changes were compared within the groups and between the groups before the operation and at 1 and 6 months.

Donor pig-specific antibodies and anti-αGal / anti-non-αGal antibody assays

Plasma concentrations of donor pig-specific IgM/IgG Abs were determined by the flow cross-match technique using donor peripheral blood mononuclear cells (PBMCs) as targeting cells.[15] Plasma levels of anti-αGal IgG and IgM Abs were measured by ELISA.[17] Their binding responses were assessed by flow cytometry using GTKO porcine endothelial cell lines (PEC69) or WT porcine endothelial cells (MPN). The level of Ab binding was determined as the net mean fluorescence intensity (nMFI). In the subjects with surviving grafts, the changes were compared both within the groups and between the groups before the operation and at 1 and 6 months.

Complement (C3a) assay

The concentration of C3a in the aqueous humor was evaluated using commercial ELISA kits (BD OptEIA™ Human C3a ELISA Kit; BD Biosciences, San Diego, CA) before the operation and at 1 and 6 months.[13,15] In subjects with surviving grafts, the changes were compared both within the groups and between the groups.

Safety monitoring

Systemic monitoring such as body weight, body temperature, complete blood count (CBC), liver function test, renal function test, and the level of glucose, was performed throughout the study, because potent immunosuppressants might cause several side effects. Reactivation of rhesus cytomegalovirus (RhCMV) was also

regularly monitored until sacrifice.

Statistical analyses

GraphPad Software (GraphPad Prism, Inc., La Jolla, CA) was used for statistical tests. Mean values of C3a concentration, T cells, Abs and endothelial cell density were compared using the Mann-Whitney U test for inter-group analysis or the Wilcoxon signed-rank test for intragroup analysis. Data are presented as the mean±standard error (SE), except for the demographics data of donors and recipients. Statistical significance was indicated by p values<0.05.

Results

Clinical outcomes

The survival duration of grafts in the anti-CD40 group was >511, >422, >273, >203, >196, and 41 days, and that of grafts in the anti-CD20 group was >470, 297, >260, >210, >184, 134, and >97 days. There was no significant difference in survival between the 2 groups (p=0.6301, log-rank test, Table 2, Figure 1 and 2). The 6-month survival rate was 83.3% (5/6) and 71.4% (5/7) in the anti-CD40 and anti-CD20 groups, respectively.

In the anti-CD40 group (Figure 1A), two primates had transparent grafts for longer than 1 year, but one recipient (anti-CD40 #4) suffered from rejection at 41 days. Corneal neovascularization appeared at 2 weeks in the first two recipients (anti-CD40 #1 and #2) and regressed spontaneously (#1) or after injecting aflibercept (#2, Figure 3). Therefore, subconjunctival aflibercept was injected to subsequent subjects, and new vessels did not develop in those grafts. Although most of the grafts were not rejected, five primates demonstrated anterior synechiae.

In the anti-CD20 group (Figure 1B), one recipient maintained a transparent graft longer than 470 days. Rejection occurred in an NHP at 134 days (anti-CD20 #2). Although one primate (anti-CD20 #3, POD 97) died due to technical accident (perforation of the colon during blood sampling), the graft was transparent until death.

The CCTs and IOPs of non-rejected NHPs were in normal ranges in both groups (Figure 4A). In rejected grafts, CCT was dramatically increased immediately before

the onset of rejection (Figure 4B). The densities of the endothelial cells were not decreased 6 months after PKP in both groups (p>0.05, Wilcoxon signed-rank test) (Figure 5). The donor-recipient junctions were well adapted, and the corneal astigmatisms were within tolerable ranges (Figure 6).

There was no infection of porcine endogenous retroviruses (PERVs) and RhCMVs in all primates (Figure 7, Table 3). Systemic adverse events that may be caused by immunosuppressants are shown in Table 4. In the anti-CD40 group, no systemic side-effects were observed. In the anti-CD20 group, 1 primate suffered from loss of appetite, and another suffered from pneumonitis, which was eventually controlled. A third primate (#6) suffered from shigellosis but recovered within one week. At 2 months, the same primate (#6) showed abnormal blood profiles that suggested disseminated intravascular coagulation (DIC) without any clinical signs. The DIC-like blood profiles were normalized after discontinuation of tacrolimus and the administration of anticoagulants and steroid.

Histology

H&E staining showed that surviving grafts in both groups had little inflammatory cell infiltration, and few lymphocytes were observed only in the donor-recipient junction (Figure 8A and 8B). However, extensive cellular infiltration and the retrocorneal membrane were observed in the rejected grafts in both groups (Figure 8C).

$CD3^+CD4^+$ T, $CD3^+CD8^+$ T, and $CD3^-CD20^+$ B lymphocytes and $CD68^+$ macrophages were barely detected in the surviving grafts, while the rejected grafts were densely infiltrated by those inflammatory cells (Figure 9).

Anti-donor pig-specific antibody responses

Subjects with surviving grafts in both groups did not exhibit an increase in the levels of anti-donor-specific IgM and IgG, except for the initial peak of IgG that was caused by non-specific IgG of IVIG as previously described.[15] (Figure 10A and 10B) There were no significant differences in the levels of IgM (p>0.05, Mann-Whitney U test; Figure 10C) and IgG (p>0.05, Mann-Whitney U test; Figure 10D) between subjects in the two groups with surviving grafts. There was also no statistically significant increase in IgM levels during follow-up in the subjects in the anti-CD40 group with surviving grafts (p>0.05, Friedman test; Figure 10C). However, in the anti-CD40 group, IgG levels significantly increased compared to the preoperative level after 1 month, but by 6 months, the levels had decreased to the preoperative level (p=0.0239, Friedman test and Dunn's multiple comparison test; Figure 10D). There were no significant changes in the anti-CD20 group (p>0.05, Friedman test; Figure 10C and 10D), but two primates with rejected grafts showed increased levels of anti-donor-specific IgG, and the level of IgG was higher in anti-CD20 #2 than in anti-CD40 #4 (Figure 10B).

Anti-Gal antibody responses and αGal expression in grafts

There were no significant differences in the levels of anti-αGal IgM and IgG and no significant changes in the mean fluorescence intensity (MFI) values of plasma samples against WT porcine endothelial cells (PECs) and GTKO PECs between subjects in the two groups with surviving grafts at 1 and 6 months (p>0.05, Mann-Whitney U test, Figure 11A and 11B), except as follows. Preoperative MFI values

against GTKO PECs were lower in the anti-CD20 group than in the anti-CD40 group (p=0.0441, Mann-Whitney test). Intragroup analysis showed that MFI values against WT PECs were significantly decreased between 1 and 6 months in the anti-CD40 group (p=0.0239, Friedman test, Figure 11B). Immunofluorescence staining demonstrated scarce expression of αGal in the surviving grafts (anti-CD40 #6 and anti-CD20 #6) (Figure 11C and 11D), whereas there was dense expression of αGal in the rejected graft (anti-CD40 #4) (x200, Figure 11E).

Changes in the concentrations of memory T and B cells in whole blood and draining lymph nodes of primates with surviving grafts

In the blood, there were no significant increases in the concentrations of interferon-gamma (IFNγ)-secreting $CD4^+$ and $CD8^+$ T cells or effector memory (EM; $CD28^-$ $CD95^+$) and central memory (CM; $CD28^+CD95^+$) T cells between the groups (Figure 12A-12F). The concentrations of B and activated B cells were significantly lower in the anti-CD20 group than in the anti-CD40 group at 1 month (Mann-Whitney test, *p* value=0.0153 and 0.0368, respectively) and 6 months (Mann-Whitney test, *p* value=0.0232 and 0.0216, respectively) (Figure 12G and 12H).

In the draining lymph nodes, there were no significant differences in the number of IFNγ-secreting $CD4^+$ and $CD8^+$ T cells or EM and CM T cells between the two groups (mean sacrifice time; anti-CD40 group POD 321±139.8 and anti-CD20 group 253±126.5) (Figure 13A, 13B, and 13C).

C3a complement in aqueous humor

In the anti-CD40 group, there were no significant increases in the concentrations of C3a at 1 and 6 months compared with the preoperative levels among subjects with surviving grafts ($p>0.05$, Friedman test; Figure 14). In contrast, in the anti-CD20 group, the level of aqueous C3a at 6 months was significantly higher than the preoperative level among subjects with surviving grafts ($p=0.0085$, Friedman test).

Discussion

Our study demonstrated that both anti-CD40 Ab-mediated blockade and the anti-CD20 Ab/basiliximab/tacrolimus regimen were effective for the long-term survival of full-thickness corneal xenotransplants in NHP studies. To our knowledge, this is the first report of a successful preclinical study of xeno-PKP with clinically available immunosuppressants. Anti-CD40 Ab-mediated blockade resulted in fewer systemic adverse reactions than the anti-CD20 Ab/basiliximab/tacrolimus regimen, although the difference was not significant. Although no cases of rejection occurred after LKP with the anti-CD40 Ab regimen,[15] PKP resulted in more cases of rejections (16.7%), suggesting that PKP is more xenogeneic. This is a reasonable result considering that the rejection rate of the allograft in PKP (which includes endothelial cells) is higher than that of the allograft in LKP (which does not include endothelial cells).[1] Consistent with previous studies,[18-20] our study also shows that corneal xenogeneic rejection is associated with both innate (complement and macrophages) and adaptive (Th1 $CD4^+$, and $CD8^+$, and B cells, which produce Gal-specific IgG Abs as well as non-Gal IgG Abs) immunities.[13] Given that CD40 is constitutively expressed on B and dendritic cells, macrophages, monocytes and eosinophils and transiently expressed on activated $CD8^+$ T cells,[21] anti-CD40 Ab-mediated blockade is effective in preventing rejection in PKP by suppressing adaptive cells as well as part of innate cells. This observation corresponds well with the other outcomes of organ transplantation using an anti-CD40 Ab combined regimen.[22,23] Taken together with our data, the use of anti-CD40 Ab can be considered as a promising option in clinical

trials of corneal xenotransplantation. The fact that 83.3% of primates experience anterior synechia with anti-CD40 Ab-based protocols indicates that low-grade inflammation may still be present in the eyes, although not at levels that would lead to graft rejection. In high-risk PKP, increased VEGF expression is known to induce corneal neovascularization,[24] and allograft rejection can be reduced with anti-VEGF treatment.[25] Considering that the anti-CD40 Ab-based protocol required treatment with an anti-VEGF trap to reduce initial neovascularization and failed to prevent synechia, unlike the anti-CD154 Ab-based protocol,[13] the anti-CD40 Ab-based protocol appears to suppress innate immunity less effectively than the anti-CD154 Ab-based protocol.

Although anti-CD40 Ab is regarded as a therapeutic candidate for human clinical trials of corneal xenotransplantation, anti-CD40 Ab-based treatment has not yet been approved by the Korean Ministry of Food and Drug Safety (KMFD). Therefore, we sought to identify alternative immunosuppressive regimens that consist of commercially available drugs approved by the KMFD and can suppress xenogeneic rejection. Rituximab (anti-CD20 Ab), tacrolimus, and basiliximab are commercially available drugs used in the allotransplantation fields.[26-29] Monoclonal antibodies (mAbs) that target specific CD proteins on the T or B cell surface (e.g., CD3, CD25, CD52) are commonly used in renal transplantation as induction therapy.[30] Compared with other mAb treatments (alemtuzumab, anti-CD20 Ab, eculizumab, muromonab-CD3, or anti-thymocyte globulin), basiliximab and daclizumab (CD25 Abs) treatments result in fewer systemic complications but have a comparable induction effect.[29,30] Therefore, basiliximab was chosen as an induction therapy. Anti-CD20 Ab and tacrolimus were selected based on previous studies that showed the

involvement of IgG Ab, $CD8^+$ T and B cells and macrophages.[6,7,13,31,32] Anti-CD20 Abs decrease the number and function of B cells by reducing (1) the activation of the complement cascade; (2) macrophage recognition, which induces phagocytosis and Ab-dependent cell-mediated cytotoxicity; and (3) natural killer cell interaction, although plasma cells are not affected. Tacrolimus is a macrolide calcineurin inhibitor, and this lipophilic compound binds to intracellular immunophilin FKBP12 (FK506 binding protein) in T lymphocytes (Figure 15). This forms a complex that prevents transcription of interleukin 2, which decreases the activation and proliferation of T lymphocytes. There is another calcineurin inhibitor, Cyclosporine, which has same mechanism to affect T lymphocytes, however it is well known that efficacy of tacrolimus is much better than that of cyclosporine. Tacrolimus is at least 10 times more potent and cause lower incidence of acute and chronic rejection than cyclosporine, and improved 1 year graft survival rate.[33-37] Therefore we choose tacrolimus rather than cyclosporine, and we were forced to exclude other medicine which are available only in oral form, not in intravenous form, such as mycophenolate mofetil and tofacitinib, because additional procedures including gastrostomy and feeding tube insertion, were needed to administer oral drugs to a rhesus and it might be harmful. Fortunately, this combination therapy (anti-CD20 Ab/tacrolimus/basiliximab) resulted in corneal graft survival for greater than 6 months in five of seven NPHs. This has satisfied the international preclinical efficacy criteria required to justify the initiation of a clinical trial. Therefore, we suggest that xenogeneic rejection of corneal transplantation may be controlled with commercially available immunosuppressive regimens.

In conclusion, both anti-CD40 Ab-mediated co-stimulation blockade and a

combination regimen (anti-CD20 Ab/basiliximab/tacrolimus) are effective for the long-term survival of full-thickness corneal xenotransplants. Especially, our results using clinically available medicine suggest that clinical trials for corneal xenotransplantation will be possible in the near future. However, considering the side-effects of the potent immunosuppressive regimens, it is necessary to adjust the dosage of immunosuppressants to reduce adverse effects, and bilateral legal blindness should be included in the selection criteria for a clinical trial.

Table 1. Demographics of donors used in the anti-CD40 and anti-CD20 Ab groups.

Anti-CD40 group				Anti-CD20 group			
Recipient	Age	Sex	ABO type	Recipient	Age	Sex	ABO type
Anti-CD40 #1	55	Male	A	Anti-CD20 #1	42	Male	A
Anti-CD40 #2	55	Male	A	Anti-CD20 #2	63	Female	A
Anti-CD40 #3	48	Male	A	Anti-CD20 #3	63	Female	A
Anti-CD40 #4	48	Male	A	Anti-CD20 #4	53	Male	A
Anti-CD40 #5	66	Female	A	Anti-CD20 #5	53	Male	A
Anti-CD40 #6	66	Female	A	Anti-CD20 #6	51	Female	A
				Anti-CD20 #7	70	Male	A
Mean ± SD 56.33±8.12				Mean ± SD 56.43±9.41			

Table 2. Demographics of recipients and graft survival with anti-CD40 and anti-CD20 Ab groups.

	Age (mo)	Sex	Body Weight (kg)	AB type	Immunosuppressive Regimen	Graft Survival (days)
Anti-CD40 1	59	Female	4.91	B	Topical prednisolone, Subconjunctival dexamethasone, Intramuscular methylprednisolone, IVIG, Intravenous anti-CD40 Ab	>203
Anti-CD40 2	59	Female	4.50	AB		>196
Anti-CD40 3	58	Female	5.02	AB		>511
Anti-CD40 4	68	Female	5.40	B		41
Anti-CD40 5	70	Female	4.82	AB		>422
Anti-CD40 6	60	Female	4.28	AB		>273
Mean ± SD	62.3±5.2		4.82±0.39			
Anti-CD20 1	63	Female	5.04	A	Topical prednisolone, Subconjunctival dexamethasone, Intramuscular methylprednisolone, IVIG, Intravenous anti-CD20 Ab, Intravenous Basiliximab, Intramuscular Tacrolimus	>260
Anti-CD20 2	64	Female	4.80	A		134
Anti-CD20 3	70	Female	4.86	AB		>97
Anti-CD20 4	71	Female	4.70	AB		>470
Anti-CD20 5	61	Female	4.80	B		297
Anti-CD20 6	113	Male	9.74	B		>210
Anti-CD20 7	55	Female	4.94	B		>184
Mean ± SD	71.0±19.3		5.55±1.85			

Table 3. Viral infections in anti-CD40 and anti-CD20 Ab groups.

	PERV		CMV	
	+	-	+	-
Anti-CD40	0 (0%)	6 (100%)	0 (0%)	6 (100%)
Anti-CD20	0 (0%)	7 (100%)	0 (0%)	7 (100%)

CMV (Cytomegalovirus)

PERV (Porcine endogenous retrovirus)

Table 4. Immunosuppressive drugs-related systemic side effects in anti-CD40 Ab and anti-CD20 Ab groups.

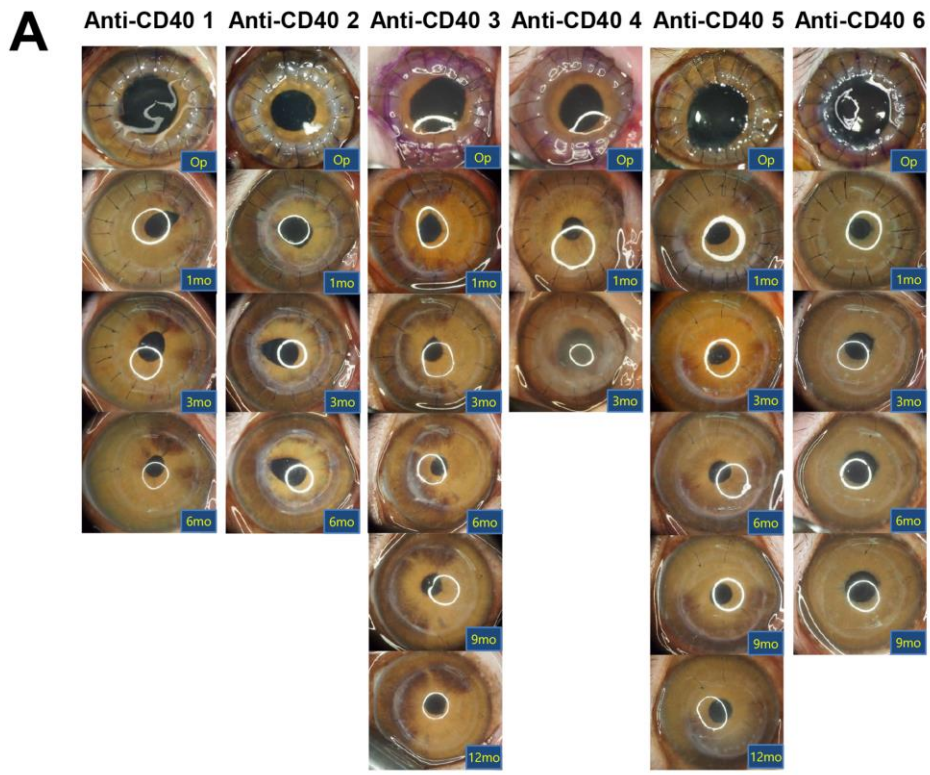
	Systemic Complication									
	Poor appetite		Diarrhea		Infection		Increased glucose level		Thrombo-cytopenic anemia	
	(+)	*p* value	(+)	*p* value	(+)	*p* value	(+)	*p* value	(+)	*p* value
Anti-CD40	0/6 (0%)	>.999	0/6 (0%)	-	0/6 (0%)	0.462	0/6 (0%)	>.999	0/6 (0%)	>.999
Anti-CD20	1/7 (14.3 %)		0/7 (0%)		2*/7 (28.6 %)		1/7 (14.3 %)		1/7 (14.3 %)	

Fisher's exact test

* Shigellosis, Pneumonitis

Figure 1. Representative serial photographs of grafted corneas in the anti-CD40 and anti-CD20 groups.

(A) All grafts in the anti-CD40 group survived longer than 6 months with one exception (anti-CD40 #4), which suffered from rejection 41 days after penetrating keratoplasty (PKP). Two primates had transparent grafts that survived longer than 1 year. (B) Five grafts in the anti-CD20 group survived longer than 6 months. One primate (anti-CD20 #2) suffered from rejection 134 days after PKP, and another primate (anti-CD20 #3) died suddenly due to an accident during sampling. However, the graft was transparent.



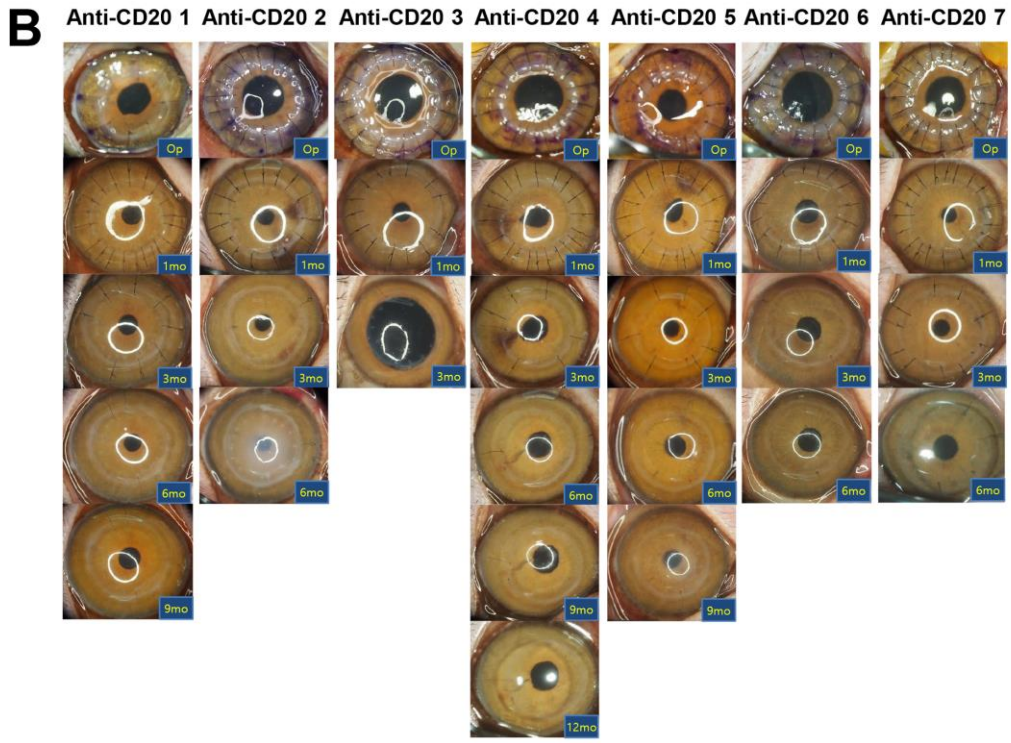

B
Anti-CD20 1
Anti-CD20 2
Anti-CD20 3
Anti-CD20 4
Anti-CD20 5
Anti-CD20 6
Anti-CD20 7
Op
1mo
3mo
6mo
9mo
12mo

Figure 2. Survival curves.

The black solid and gray dotted lines represent graft survival in the anti-CD40 and anti-CD20 groups, respectively. The survival times of the anti-CD40 group (n=6) were >511, >422, >273, >203, >196, and 41 days, and those of the anti-CD20 group (n=7) were >470, 297, >260, >210, >184, 134, and >97 days. There was no difference in survival between the two groups (p=0.630, log-rank test).

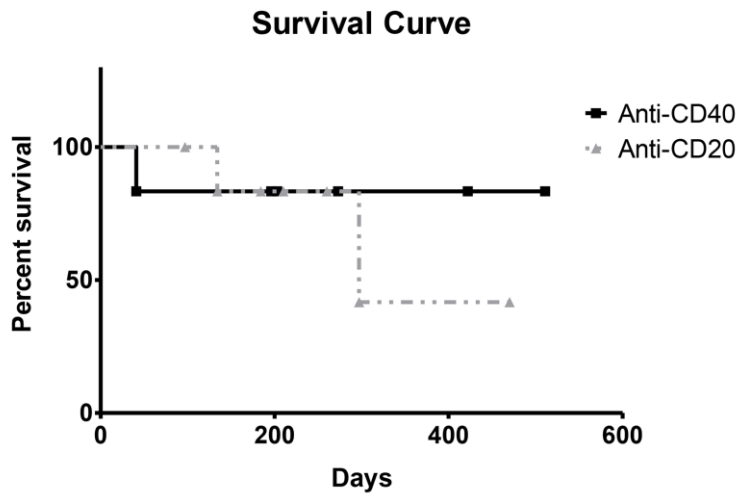


Figure 3. Corneal neovascularization in the anti-CD40 group.

In anti-CD40 #2, neovascularization crossed over the junction and reached the periphery of the donor cornea (A, arrows indicate neovascularization), although it did not induce rejection. The new vessels regressed after subconjunctival injection of aflibercept (B)

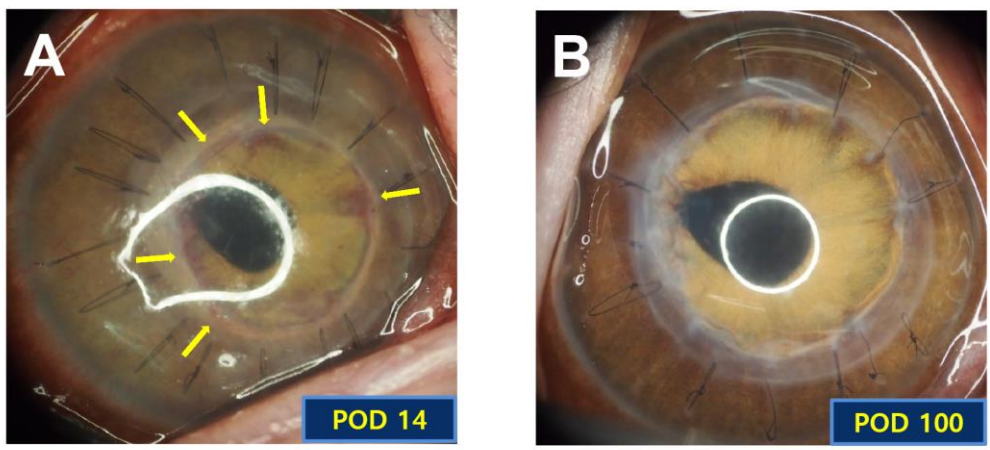


Figure 4. Changes in mean central corneal thickness (CCT) and intraocular pressure (IOP) in the anti-CD40 and anti-CD20 groups.

(A) CCT and IOP were well maintained within normal ranges in recipients with surviving grafts during the follow-up period. (B) In rejected grafts, CCT was dramatically increased immediately before the onset of rejection. Horizontal gray dotted lines and arrow indicate the time point that the rejection was defined.

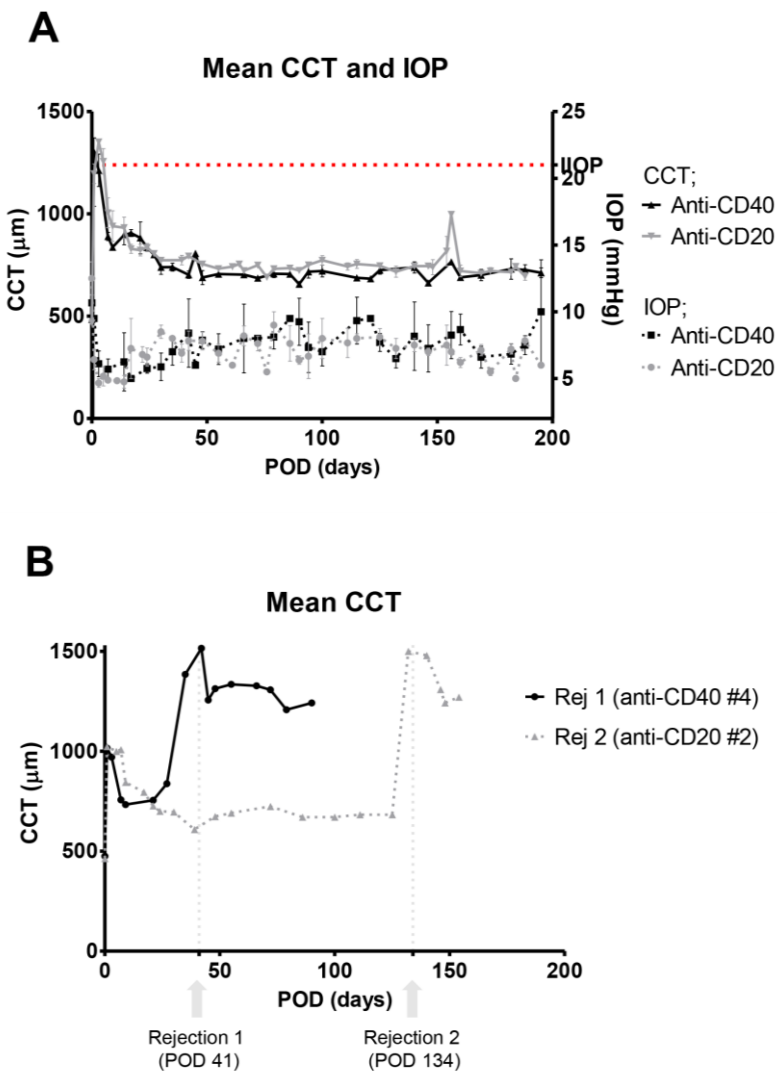


Figure 5. Representative images of endothelial cells and average endothelial cell density (ECD) in the surviving grafts.

(A) The ECD of a surviving graft in the anti-CD40 group (anti-CD40 #6, postoperative day (POD) 273) was well maintained, with a cell density of 1553 cells/mm^2 accompanied by normal ranges of coefficient of variance (CV; 32) and hexagonality (HEX; 65). (B) The ECD of a surviving graft in the anti-CD20 group (anti-CD20 #1, POD 260) was well maintained, with a cell density of 2075 cells/mm^2 accompanied by normal ranges of CV (46) and HEX (54). (C) Average changes in ECD in grafts after sacrifice were not significantly different compared to the preoperative ECD (mean±standard error; p>0.05, Wilcoxon signed-rank test). Only the primates with available pre- and postoperative data were included (n=10).

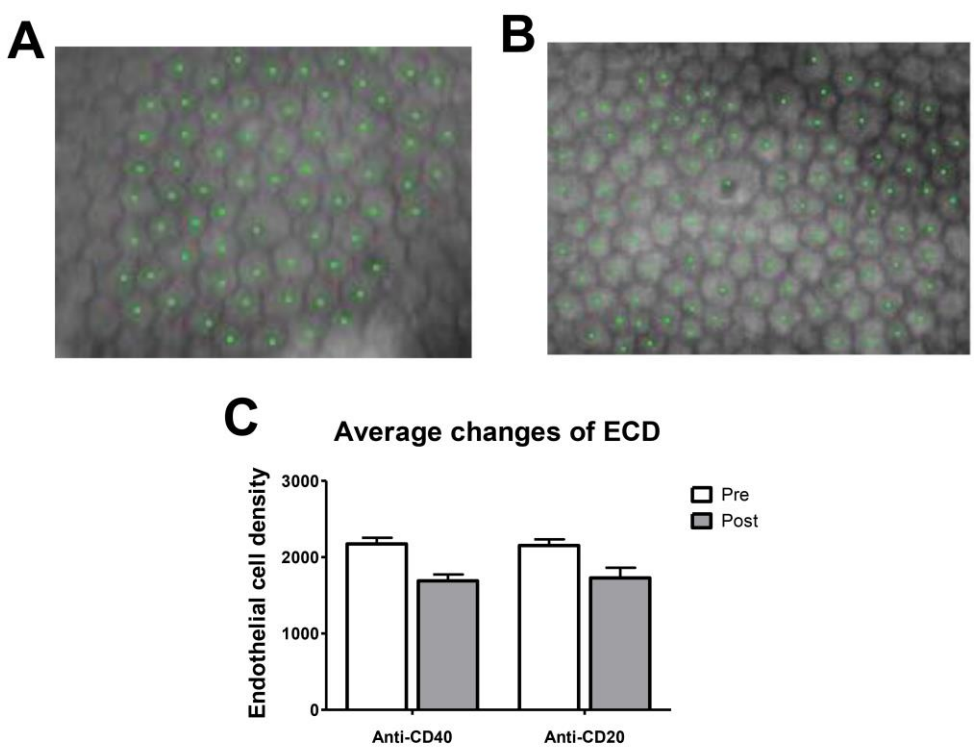


Figure 6. Representative topography and anterior segment optical coherent tomography of surviving grafts in both groups.

(A, B) Simulated K astigmatism is shown by topography. (C, D) Anterior segment optical coherent tomography demonstrated a well approximated wound and normal depth of the anterior chamber.

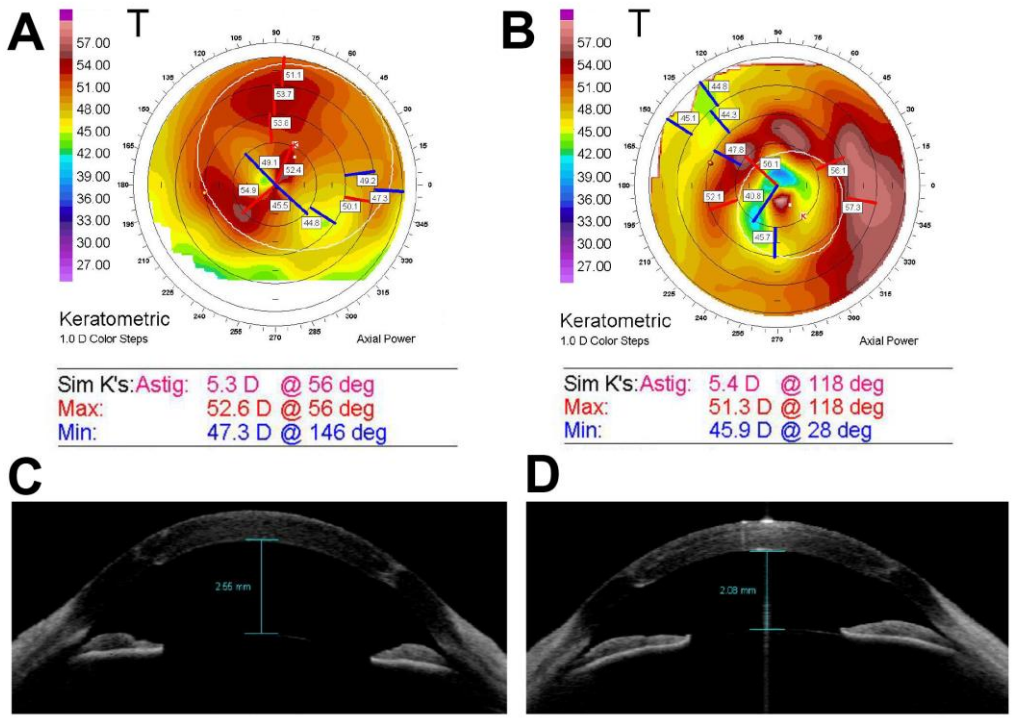
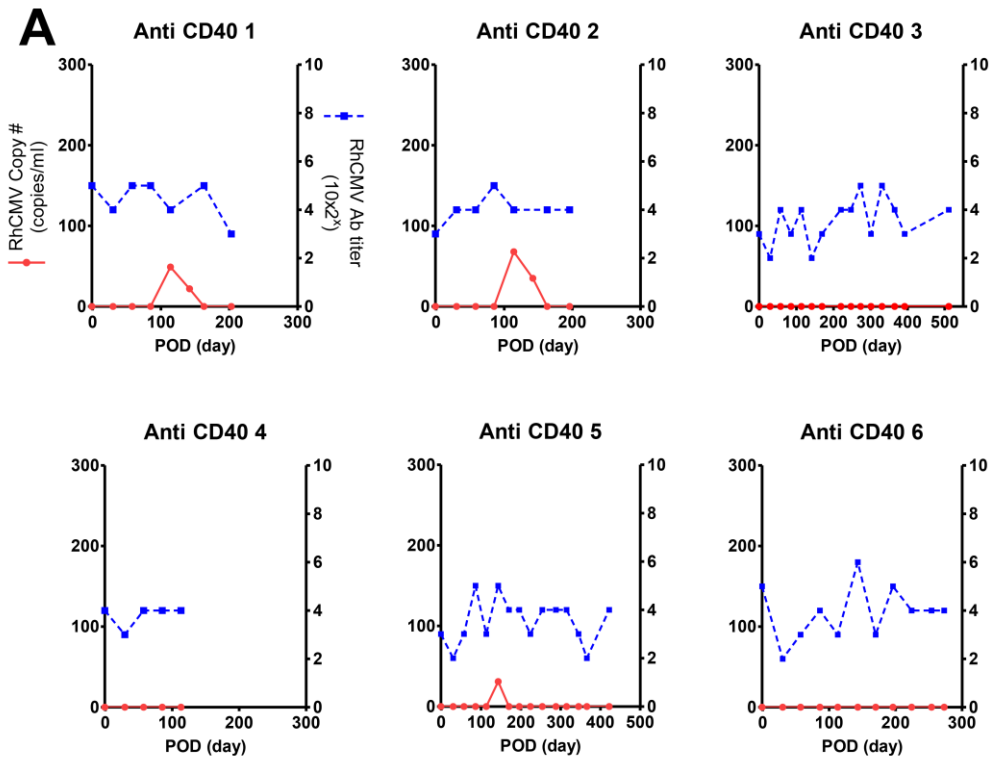


Figure 7. Monitoring of Rhesus cytomegaloviruses (RhCMVs) in peripheral blood of recipients after pig-to-rhesus penetrating keratoplasty.

(A, B) In all recipients, the copy numbers of DNA (red solid line) in plasma and the anti-RhCMV antibody responses (blue dotted line) did not increase, which suggested that CMV reactivation did not occur in both the anti-CD40 group (A) and anti-CD20 group (B).



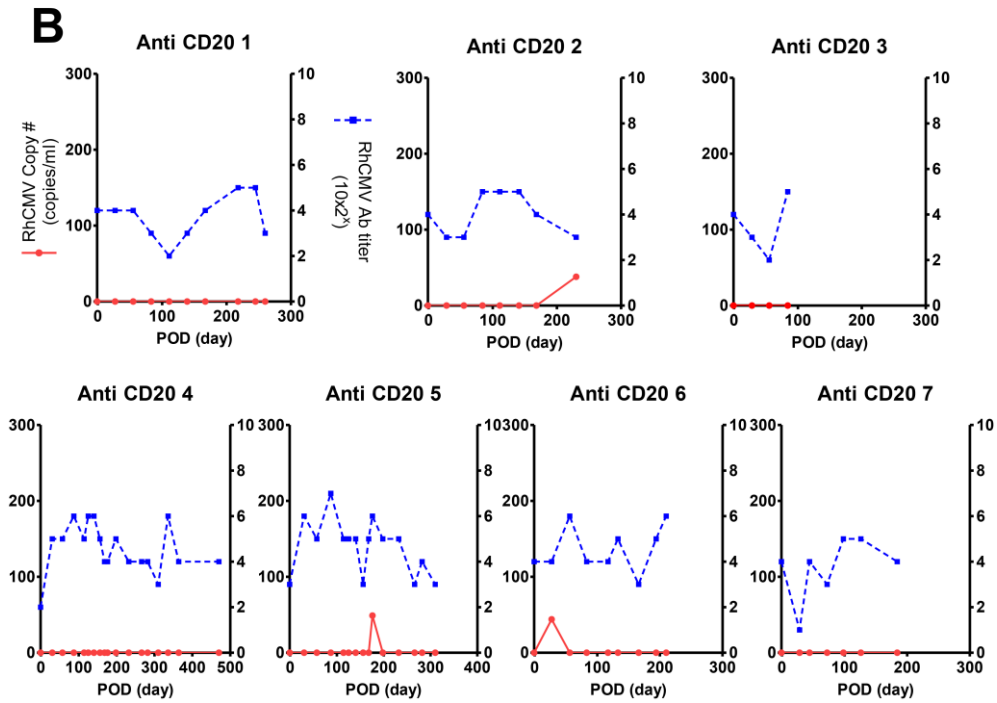
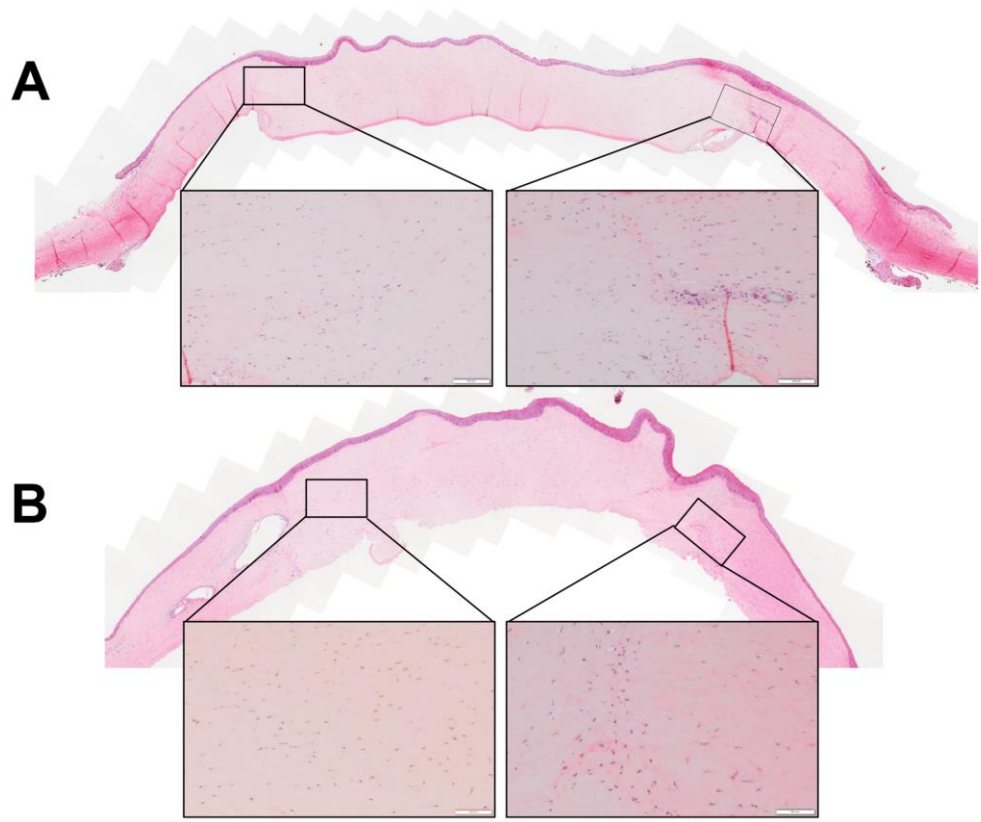

B
Anti CD20 1
Anti CD20 2
Anti CD20 3
Anti CD20 4
Anti CD20 5
Anti CD20 6
Anti CD20 7
RhCMV Copy # (copies/ml)
RhCMV Ab titer (10x2^x)
POD (day)

Figure 8. Representative hematoxylin & eosin (H&E) staining images of surviving grafts in each group and rejected porcine corneal grafts.

(A) Staining of a surviving graft in the anti-CD40 group (anti-CD40 #2, postoperative day (POD) 196) showing mild infiltration of inflammatory cells limited to the donor-recipient junction. (B) Staining of a surviving graft in the anti-CD20 group (anti-CD20 #1, POD 260) closely resembling the surviving graft in (A). (C) Staining of a rejected graft in a primate (anti-CD40 #4, POD 113) showing graft edema and dense infiltration of inflammatory cells in the entire graft (a), including the donor-recipient junction (b). In addition, the retrocorneal membrane, which was not found in the surviving grafts, was observed in the rejected graft (c and d).



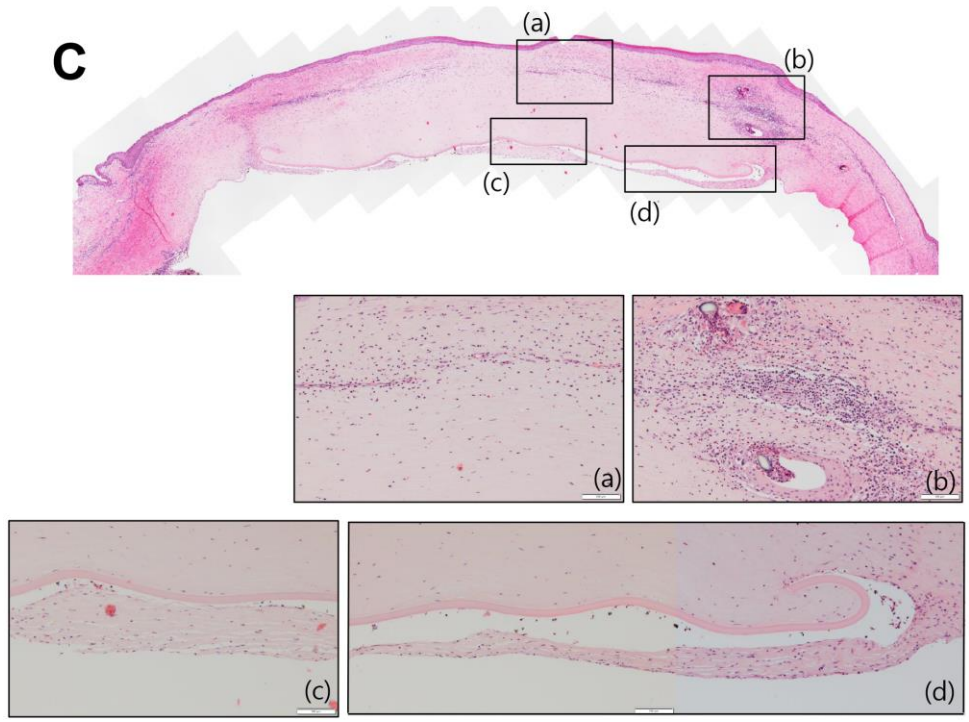

C
(a)
(b)
(c)
(d)
(a)
(b)
(c)
(d)

Figure 9. Immunofluorescence staining of the rejected grafts.

Rejected grafts were densely infiltrated by inflammatory cells such as $CD3^+CD4^+$ T, $CD3^+CD8^+$ T, and $CD3^-CD20^+$ B lymphocytes and $CD68^+$ macrophages (upper panel; anti-CD40 #4, POD 113, lower panel; anti-CD20 #3, POD 229) (X200).

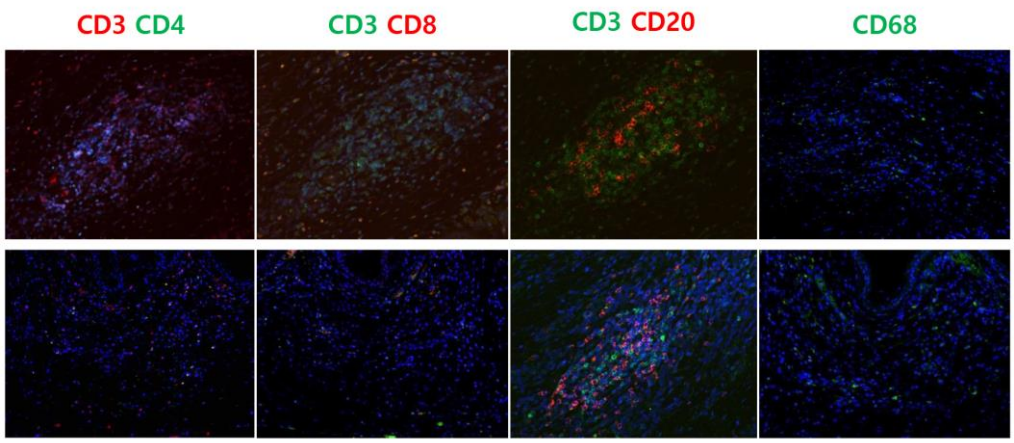


Figure 10. Changes in plasma donor pig-specific IgM and IgG for 6 months.

(A, B) In the plasma of recipients in both groups who had surviving grafts, the levels of anti-donor-specific IgM and IgG did not increase, except for the initial peak of IgG that was caused by binding of non-specific IgG of intravenously injected immunoglobulin. Two primates with rejected grafts showed increased levels of anti-donor-specific IgG, and the level of IgG was higher in anti-CD20 #2 than in anti-CD40 #4. (C, D) There were no significant differences in the levels of IgM and IgG between subjects in the two groups with surviving grafts at preoperation and at postoperative 1 and 6 months (p>0.05, Mann-Whitney test). Although the levels of IgG were significantly increased in the anti-CD40 group at 1 month compared to the preoperative level (p=0.0239, Friedman test and Dunn's multiple comparison test), the levels had decreased to the preoperative level at postoperative 6 months.

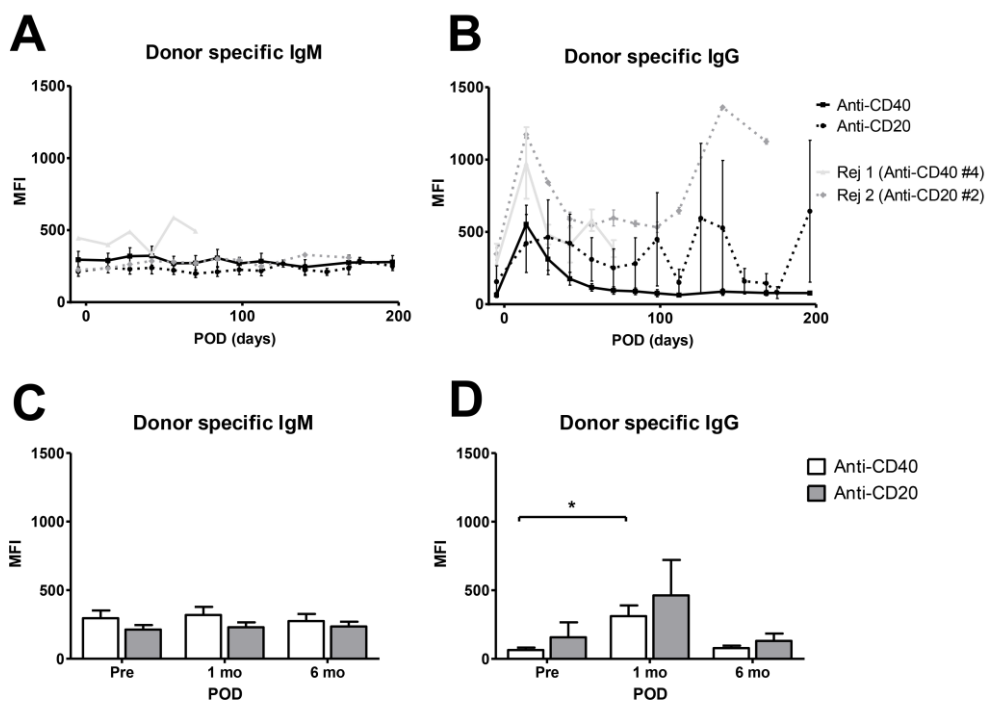


Figure 11. Changes in plasma anti-αGal and non-Gal antibodies and αGal expression in porcine corneal grafts.

(A, B) There were no significant differences in the levels of anti-αGal IgM and IgG and no significant changes in the mean fluorescence intensity (MFI) values of plasma samples against wild-type (WT) porcine endothelial cells (PECs) and GTKO PECs between subjects in the two groups with surviving grafts at postoperative 1 and 6 months (p>0.05, Mann-Whitney U test) except as follows. Preoperative MFI values against GTKO PECs was lower in the anti-CD20 group than in the anti-CD40 group (p=0.0441†, Mann-Whitney test). In the intragroup analysis, MFI values against WT PECs was significantly decreased between 1 and 6 months in the anti-CD40 group (p=0.0239*, Friedman test). (C, D) Representative figures of the immunofluorescent staining of αGal. Low levels of αGal were expressed in surviving grafts (anti-CD40 #6 and anti-CD20 #6). (E) In contrast, there was a high expression level of αGal in the rejected graft (anti-CD40 #4) (x200). Yellow arrows indicate αGal staining in the cells.

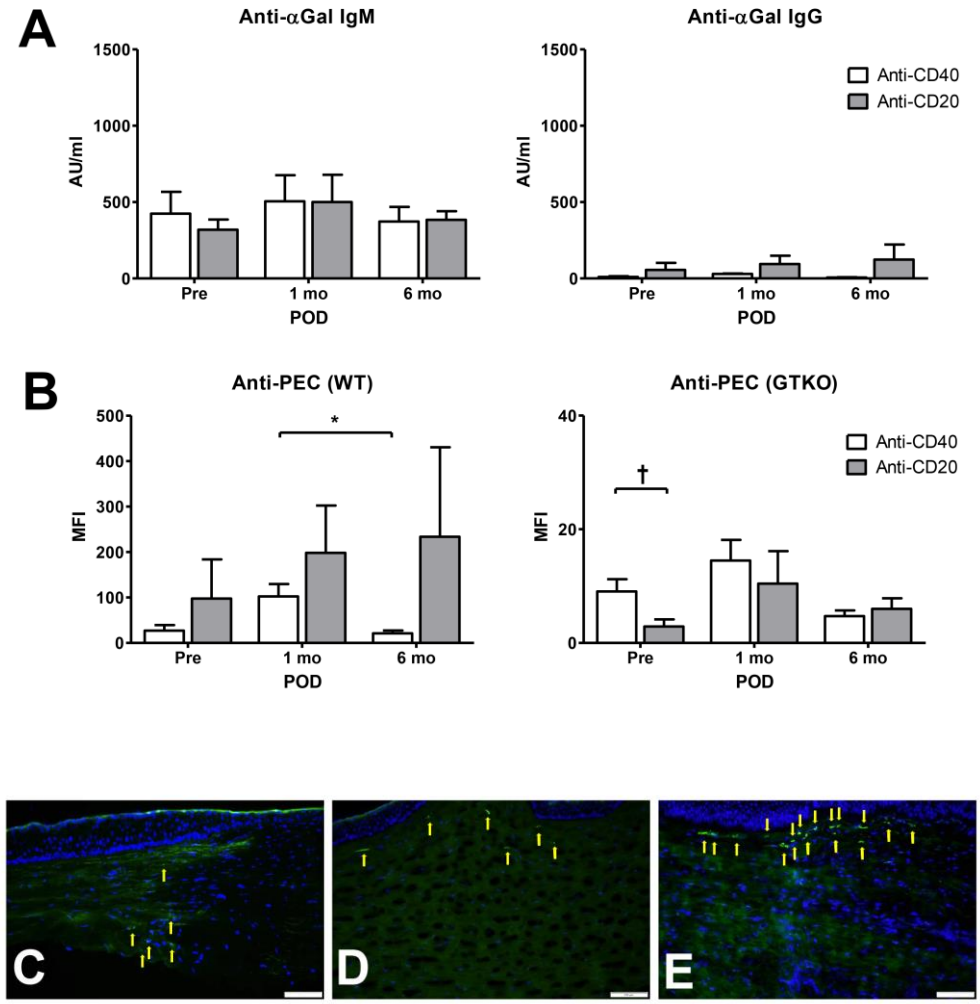

A
Anti-αGal IgM
Anti-αGal IgG
AU/ml
1500
1000
500
0
Pre
1 mo
6 mo
POD
Anti-CD40
Anti-CD20
B
Anti-PEC (WT)
Anti-PEC (GTKO)
MFI
500
400
300
200
100
0
40
20
*
†
C
D
E

Figure 12. Changes in the concentrations of T cells and B cells in blood of the anti-CD40 and anti-CD20 groups.

(A-F) There was no significant increase in the concentrations of interferon-gamma (IFNγ)-secreting $CD4^+$ and $CD8^+$ T cells or effector memory (EM; $CD28^-CD95^+$) and central memory (CM; $CD28^+CD95^+$) T cells at postoperative 1 and 6 months compared with their preoperative levels in both groups. (G, H) There were significant decreases in the concentrations of B cells and activated B cells in the anti-CD20 group at 1 month compared with the preoperative levels (p=0.0239* and 0.0085†, respectively, Friedman test, Dunn's multiple comparison test). In addition, in the recipients with surviving grafts in the anti-CD20 group, the concentrations of B cells and activated B cells were significantly lower than those in the anti-CD40 group at postoperative 1 month (p=0.0153‡ and 0.0368§, Mann-Whitney U test) and 6 months (p=0.0232$^\#$ and 0.0216**, Mann-Whitney U test).

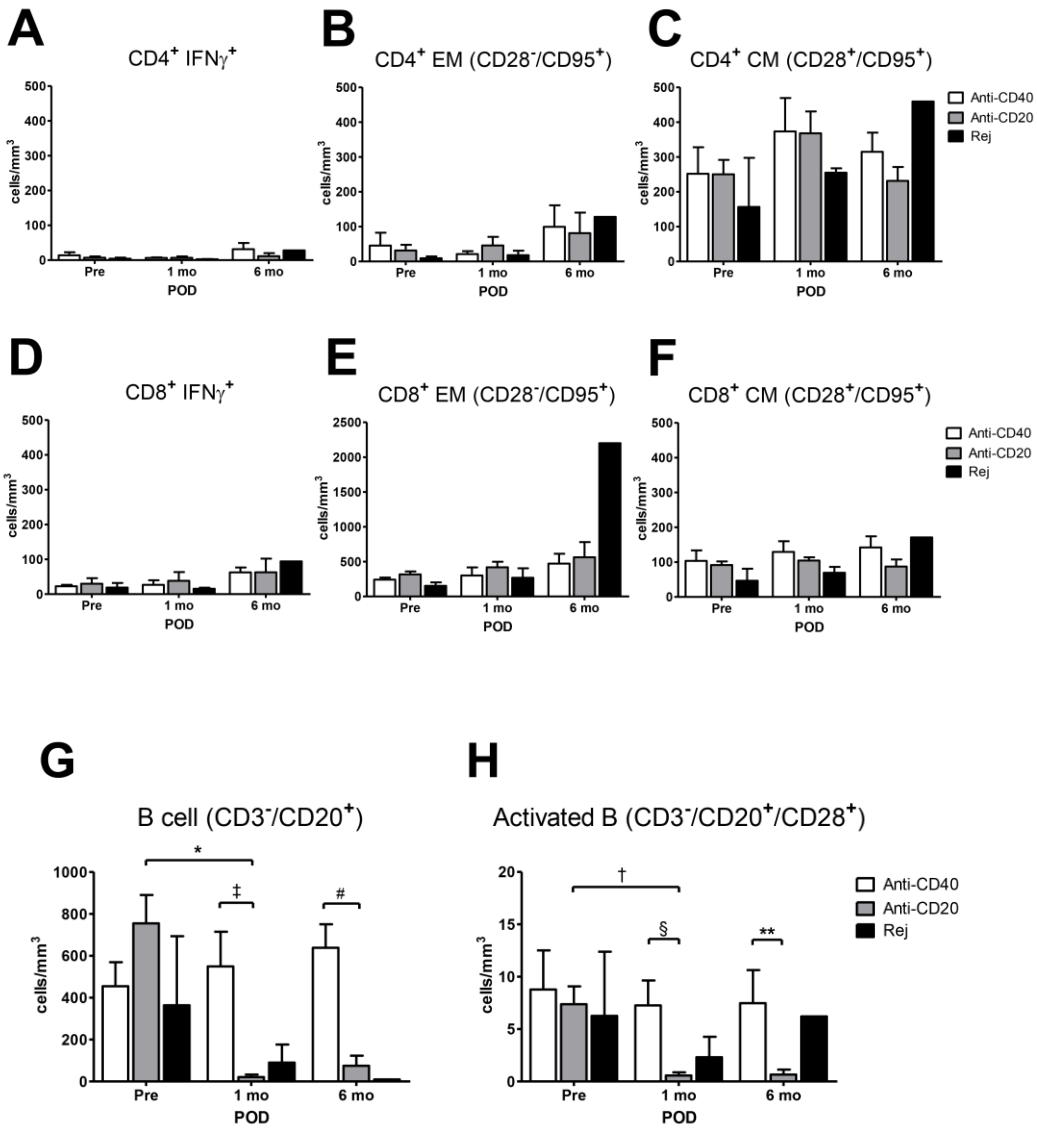

A
CD4+ IFNγ+
B
CD4+ EM (CD28-/CD95+)
C
CD4+ CM (CD28+/CD95+)
D
CD8+ IFNγ+
E
CD8+ EM (CD28-/CD95+)
F
CD8+ CM (CD28+/CD95+)
cells/mm3
POD
Pre
1 mo
6 mo
Anti-CD40
Anti-CD20
Rej
G
B cell (CD3-/CD20+)
H
Activated B (CD3-/CD20+/CD28+)
*
‡
#
†
§
**

Figure 13. Comparison of the concentration of T cells in draining lymph nodes between the anti-CD40 and anti-CD20 groups.

(A-C) There were no significant differences in the number of IFNγ-secreting $CD4^+$ and $CD8^+$ T cells or EM and CM T cells between the 2 groups at the time of sacrifice. (p>0.05, Mann-Whitney U test). All data were analyzed after the subjects were sacrificed (mean sacrifice time; anti-CD40 group POD 321±139.8 and anti-CD20 group POD 253±126.5).

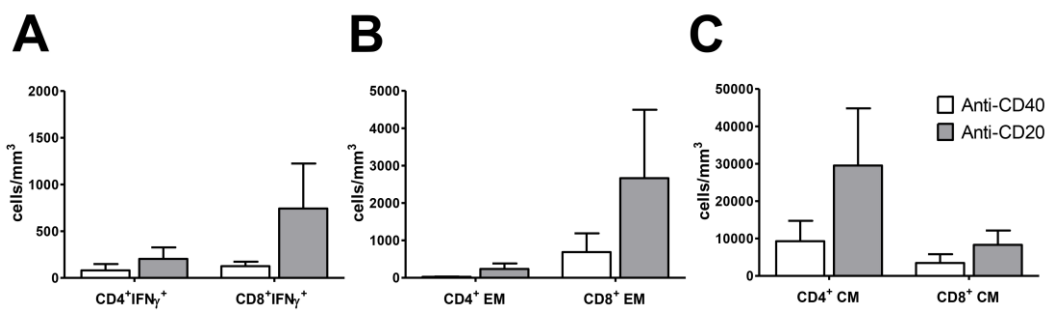


Figure 14. Changes in the concentration of aqueous humor complement (C3a) in the anti-CD40 and anti-CD20 groups.

There were no significant increases in the levels of C3a in aqueous humor at 1 and 6 months compared to the preoperative levels in recipients with surviving grafts in the anti-CD40 group (p>0.05, Friedman test). In contrast, the concentration of aqueous C3a increased significantly at 6 months compared to the preoperative level in recipients with surviving grafts in the anti-CD20 group (p=0.0085**, Friedman test).

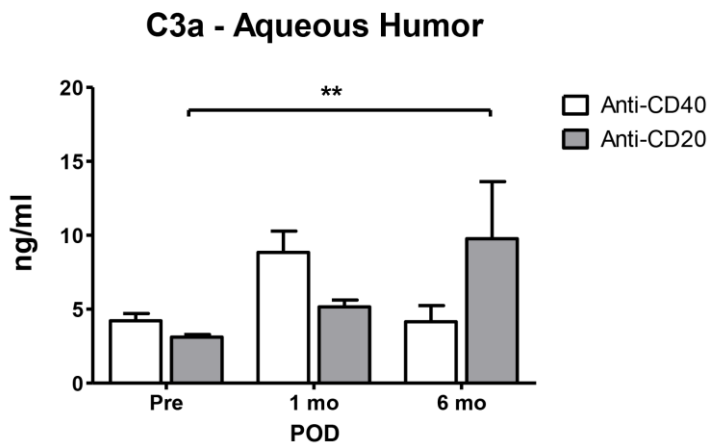
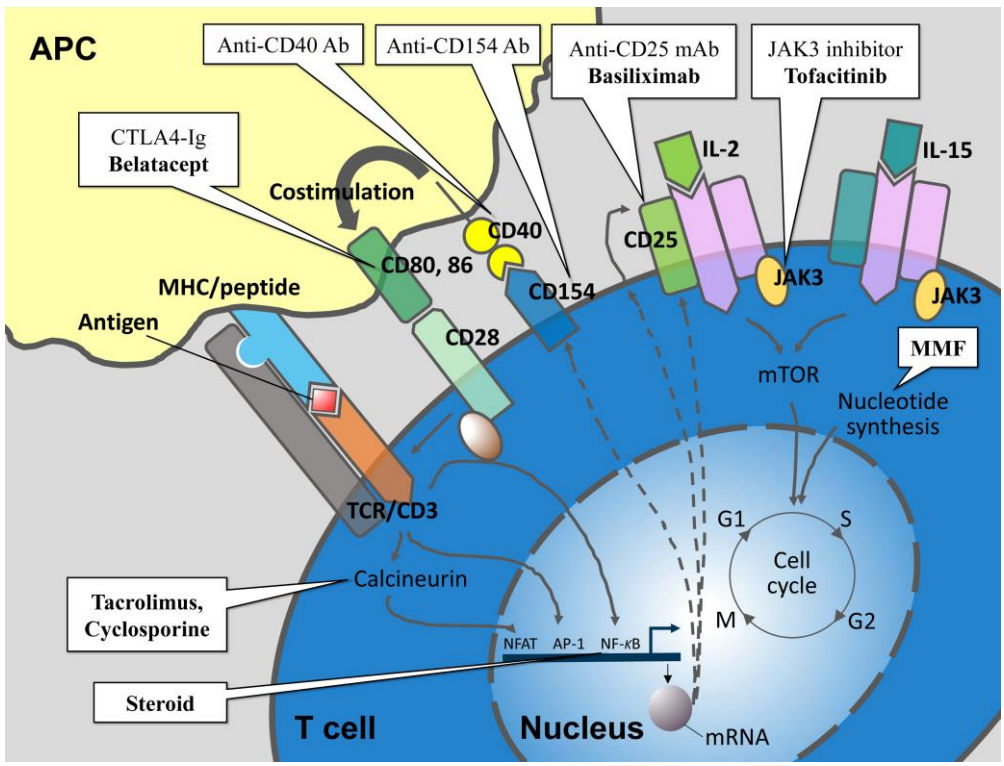


Figure 15. Immunosuppressants that target T lymphocytes

There are several immunosuppressants targeting T lymphocytes. Among them, we choose some drugs such as Basiliximab, Tacrolimus, and steroid that commonly used in allo-organ transplantation already and that are available in intravenous forms in Korea.



Acknowledgments

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국문 초록

돼지-영장류 간 이종 전층각막이식에서 항 CD40 단일클론항체를 이용한 면역억제요법과 항 CD20 단일클론항체 및 타크로리무스를 포함한 복합면역억제요법의 장기 유효성과 안전성의 비교 분석

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목적: 인구의 노령화와 백내장 수술, 굴절교정 수술 등의 보편화로 인해 각막을 기증할 수 있는 기증자의 수가 점차 줄어들고 있다. 돼지의 각막은 인간의 각막을 대체할 수 있는 적절한 후보 물질이며, 이를 이용한 이종각막이식이 인간 공여각막의 부족 문제를 해결할 수 있는 효과적인 대안으로 활발히 연구되고 있다. 이러한 이종각막이식을 성공적으로 시행하기 위해서는 이종간의 항원 차이를 극복하기 위한 면역억제제의 사용이 필수적이다. 본 연구에서는 돼지-영장류 간 이종 전층각막이식에서 항 CD40 항체를 이용한 공자극 차단 면역억제요법과 현재 임상에서 사용 가능한 항 CD20 항체를 비롯한 바실리시맙, 타크로리무스의 복합

면역억제요법의 장기적인 효과 및 안전성을 비교하여, 향후 임상시험에서 사용 가능한 면역억제요법을 선정하고자 한다.

방법: 열 세 마리의 영장류 (Chinese rhesus macaques) 에서 7.5mm 직경의 돼지 각막을 이용하여 전층각막이식을 시행하였다. 이 중 여섯 마리는 항 CD40 항체(2C10R4)를 계획된 일정대로 정맥 투여 하였고, 나머지 영장류에는 항 CD20 항체, 바실리시맙 및 타크로리무스를 정맥 혹은 근육 주사로 투여하였다. 이식 각막편의 생존여부를 관찰하기 위해, 정기적으로 이식 각막편을 관찰하였고, 중심 각막 두께와 안압을 측정하였다. 또한 영장류 혈액 내의 작동 및 기억 T 세포 및 B 세포, 공여자 특이 항체, 항 αGal 항체 및 방수 내 보체의 변화를 조사하였고, 지속적인 약제 투여와 장기간의 면역억제로 인한 전신 부작용의 발생 여부를 관찰하였다.

결과: 항 CD40 항체를 투여한 군과(511, 422, 273, 203, 196일 이상, 41일) 항 CD20 항체 등을 투여한 군(470, 260, 210, 158, 97일 이상, 297, 134일) 모두에서 이식 각막편의 장기 생존을 관찰하였다. 항 CD20 항체 등을 투여한 군에서 활성 B 세포의 수가 항 CD40 항체 투여 군에 비해 유의하게 낮았고 (p=0.0216, Mann−Whitney test), 수술 후 6개월 째에 방수 내 보체의 농도가 수술 전과 비교하여 유의하게 높았다 (p=0.0085, Friedman test). 항 CD20 항체 등을 투여한 영장류

중 세 마리에서 전신 부작용이 관찰되었으며, 이는 모두 일시적인 약물 치료로 조절되었다.

결론: 항 CD40 항체를 이용한 공자극 차단 면역억제요법과 항 CD20 항체등의 복합면역억제요법은 모두 돼지-영장류 간 이종 전층각막이식에서 이식 각막편의 장기 생존에 효과적이었으나, 항 CD20 항체 등을 투여한 군에서 일시적인 전신 부작용을 보인 개체가 있었다. 항 CD20 항체 등의 복합면역억제요법은 현재 임상에서 사용 중인 약제들로 이종 각막이식의 임상시험에 바로 적용이 가능하다는 장점이 있으나, 보다 안전하게 사용하기 위해서는 부작용을 최소화하기 위한 용량 조절이 반드시 필요할 것으로 사료된다. [1]

주요어: 항 CD40 항체, 항 CD20 항체, 바실리시맙, 타크로리무스, 각막, 전층각막이식, 영장류, 이종이식

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[1] 본 박사학위 논문은 American Journal of Transplantation (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.) 에 출판 완료된 내용을 포함하고 있습니다.

감사의 글

사람은 살면서 세 번의 기회가 있다고 합니다. 제게 첫 번째 기회는 전공을 바꾸어 의학을 공부한 것이고, 두 번째 기회는 서울대학교병원 안과에서 전안부 임상강사로 수련을 하게 된 것이라고 생각합니다. 안과 전문의 자격증을 갓 취득한 뒤, 연구나 임상 경험이 턱없이 부족했던 제게 서울대학교병원이라는 거대하고 낯선 곳에서의 새로운 시작은 큰 용기가 필요한 일이었습니다. 그러나 저를 따뜻하게 받아주신 위원량, 김미금, 오주연 교수님과의 만남은 제 인생의 터닝 포인트가 되었습니다. 오랜 임상 경험과 지식에서 나오는 연륜으로 늘 좋은 말씀을 들려주시고 멋진 리더의 모습을 손수 보여주시는 위원량 교수님, 잘할 때나 잘못할 때나 엄마 같은 마음으로 가르쳐 주시고, 좋은 일, 슬픈 일 모두 당신의 일처럼 함께 해 주시는, 제가 아는 가장 좋은 의사이시고, 훌륭한 연구자이자, 따뜻한 선배님으로 모든 것을 배우고 싶은 김미금 교수님, 그리고 너무 멋있어 닮고 싶고, 항상 언니처럼 든든하고, 세상에서 제일 쿨하시면서도 보이지 않게 마음 써 주시는 오주연 교수님을 만난 것은 제 인생에 가장 큰 행운입니다. 세 분 선생님은 제 평생의 은사님으로 사랑과 존경의 마음을 가슴 깊이 간직하고 있습니다. 항상 감사드립니다. 분당서울대병원의 현준영, 전현선 교수님, SNU청안과의 한영근 원장님도 제가 이 곳에 정을 붙이고, 전안부를 전공하는 것에 자부심을 가질 수 있도록 도와주신 감사한 분들입니다. 뒤늦게 만나 뵈었지만, 실질적으로 본 연구가 있을 수 있도록 이전의 연구들을 성공적으로 이끌어주신, 그리고 지금은 전안부 선배님으로 큰 힘이 되어주시는 서울대학교병원 최혁진 교수님께도 감사드립니다.

이 연구는 십 여년의 시간동안 기초적인 부분부터 하나하나 쌓아 올려진 공든탑의 정점에 있는 연구라 생각됩니다. 긴 시간 앞선 연구들을 꼼꼼하게 설계하시고, 끈질기게 이어오신 서울대학교병원 안과의 전안부 (코니안즈) 선배님들의 노고가 있었기에 가능할 수 있었습니다. 모든 선배님들께 진심으로 감사 드리며, 지금의 영광을 그분들께 돌립니다.

또한 이 연구는 저 혼자 힘으로는 절대 해낼 수 없는 아주 큰 규모의 어려운 프로젝트였습니다. 전임상 실험이었기 때문에 많은 동물들을 접해야 했고, 유효성과 안전성 규명을 위한 실험들을 수없이 거쳐야 했기에, 많은 분들의 도움이

없었다면 불가능했을 것입니다. 항체와 보체 검사를 도와주신 한림대학교 성심병원 진단검사의학과 강희정 교수님과 PERV 및 CMV 검출에 도움 주신 서울대학교 의과대학 미생물학교실 황응수 교수님, 모든 실험을 도와주시고, 가장 가까이에서 저를 보살펴 주신 안면역재생연구실의 이현주, 류진숙 연구원님, 처음부터 끝까지 매일을 하루같이 영장류들에 약제를 투여하고, 꼼꼼하게 컨디션을 점검해준, 성실하고 심성 고운 권현상 연구원님, 이분들 덕분에 본 연구를 성공적으로 완료할 수 있었습니다. 진심으로 감사합니다. 영장류들의 사육 및 관리부터 모든 수술이 안전하게 진행되도록 도와주시고, 긴 연구기간 내내 수의학적 자문을 아끼지 않으신 서울대학교 이종장기사업단의 영장류팀 김종민 박사님과 연구원님들, 무균돼지의 처치와 안구적출 등의 과정에서 항상 안과팀을 도와주시고, 이종이식 전반에 대한 지식과 경험의 공유, 그리고 따뜻한 조언으로 전폭적인 지지를 보내주신 췌도팀의 신준섭, 민병훈 박사님과 연구원님들께 깊은 감사를 드립니다. 연구 전반을 지휘하시면서 안과팀에 응원과 지원을 아끼지 않으신 이종장기사업단의 박정규 단장님, 최병선 국장님과 조지은, 이우림 사무원님, 보이는 곳, 보이지 않는 곳에서 도움 주신 김현제, 김용희 박사에게도 감사 인사 드립니다. 이 분들이 계셨기에 병원 생활을 하면서도 이런 막중한 프로젝트를 성공적으로 진행할 수 있었고, 큰 사고 없이 즐겁게 일하고, 소중한 경험과 추억을 쌓을 수 있었습니다. 이 모든 시간 동안 옆에서 같이 고민하고, 함께 헤쳐나간 제 동기 김유정 교수와 안과 동반자이자 모든 일상을 함께하는 최세랑, 문지영, 김민경 원장에게도 사랑과 감사를 전합니다.

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오랜 벗 현진, 효진, 장은, 고은, 베프 유지연, Lucid 보영, 미희, 윤미, 원정, 소희, 미리, 혜아, 윤주, Repose 지현, 숙희, 민아, 슬기, AOZ 은경, 지명, 미유, 상은, 희진, 멋진 이은정, 동기 강준원, 우리언니 최미영, 어린 시절 제 롤모델이셨고, 항상 올바른 길을 제시해주시는 열정의 아이콘 조민정 도덕 선생님, 힘

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아직도 너무 많이 부족하지만, 제가 받은 모든 감사한 기회, 격려와 도움 잊지 않고, 좋은 의사, 좋은 학자가 될 수 있도록 더욱 정진하고, 노력하겠습니다.

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