Immune Cells on the Corneal Endothelium of an Allogeneic Corneal Transplantation Rabbit Model

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Purpose. Corneal endothelial cell density undergoes a progressive decrease for many years after transplantation, eventually threatening patients with late endothelial failure. The purpose of this study was to investigate the possibility of an immunologic response in successfully grafted corneal endothelium.

Methods. The corneal endothelium of patients who had undergone corneal transplantation was evaluated by specular microscopy. Rabbit models were subjected to penetrating keratoplasty (PK) with either syngeneic or allogeneic corneal transplants and Descemet's stripping endothelial keratoplasty (DSEK) with allogeneic corneal transplants. The presence of immune cells and expression of proinflammatory cytokines were determined by immunostaining. The corneal endothelium and immune cells were also evaluated by scanning electron microscopy.

Results. Scanning slit contact specular microscopy of patients with no features of graft rejection revealed cell-like white dots on the grafted corneal endothelium. The corneal endothelium of the allogeneic PK and DSEK rabbit models displayed the presence of immune cells, including $CD4^+$ T-helper cells, $CD8^+$ cytotoxic T cells, $CD20^+$ B lymphocytes, $CD68^+$ macrophages, and neutrophils, but these immune cells were rarely observed in the syngeneic PK model. These immune cells also produced proinflammatory cytokines. Notably, some of the corneal endothelial cells situated near these immune cells exhibited features of apoptosis.

Conclusions. T lymphocytes, B lymphocytes, macrophages, and neutrophils are present on the grafted corneal endothelium in both PK and DSEK allogeneic rabbit models. The potential involvement of immune cells as an underlying pathophysiology for late endothelial failure deserves further examination.

Keywords: corneal endothelium, immune cells, inflammation

Corneal transplantation is one of the most common transplantations performed in the world.[1] The Eye Bank Association of America[2] reported that 47,530 donor corneas were provided for transplantation in the United States; similarly, 28,901 donor corneas were provided for transplantation in other countries in 2014. Corneal transplantation has been accepted as a successful procedure, but several large cohort studies have now shown that graft survival after 10 years following penetrating keratoplasty (PK) is only approximately 50%,[3–5] although this varies widely depending on the indications and the specific studies.[6,7] Endothelial dysfunction also accounts for 50% of the total graft failures, suggesting that corneal endothelial damage is the leading cause of graft failure.[5,7]

Endothelial graft failure can be divided into three types: primary graft failure (mainly iatrogenic), immunologic rejection, and late endothelial failure.[6] Graft rejection is clinically recognized by features that include the presence of endothelial rejection line, keratic precipitates, corneal edema, and conjunctival injection caused by immunologic responses against donor cells.[8–10] One multicenter study revealed that 92 of 1090 patients (8.4%) experienced graft failure within 5 years of follow-up due to rejection after PK. On the other hand, late endothelial failure typically exhibits no clinical symptoms, but several studies, including a large cohort study based on graft registries, have demonstrated that corneal endothelial cell (CEC) density decreases continuously, even in clinically successful transplantation cases. For instance, the Cornea Donor Study Research Group reported that patients who underwent PK experienced a cell loss of 79%, resulting in a median 10-year corneal endothelial cell density (ECD) of 550 cells/mm^2.[7]

The damage to the corneal endothelium associated with ECD drop is compensated by increased pump function and barrier function by remaining CECs, which maintains corneal transparency.[11] However, a decrease in the ECD to a critical level, typically 500 cells/mm^2, overwhelms this ability to compensate, and the cornea begins to exhibit corneal edema with severe visual disturbance. This suggests that graft corneas, on average, come close to the threshold ability to maintain corneal transparency or exhibit haziness within a decade. Late

endothelial failure is accepted as a leading threat of graft failure, but the underlying mechanism remains unclear and no treatment is available. Hence, a better understanding of the source of this failure is urgently needed, not only for understanding the pathophysiology, but also for developing preventive treatments for late endothelial failure.

Birnbaum and colleagues[12] reported that the relative annual loss of endothelial cells was 1.1% after autologous rotational keratoplasty, whereas the relative annual loss was 16.7% in homologous PK.[12] Only six patients were analyzed as the autologous rotational keratoplasty group, but these authors hypothesized that immunologic influences might be the cause of the chronic endothelial cell loss observed after corneal transplantation.[12] We pursued this idea by investigating the potential involvement of an immunologic response in the CECs of corneal grafts that are clinically transparent without rejection symptoms after transplantation.

In this study, we explored the possibility of an immunologic response in the corneal endothelium in a rabbit corneal transplantation model. We showed that immune cells, such as $CD4^+$ T-helper cells, $CD8^+$ cytotoxic T cells, $CD20^+$ B lymphocytes, $CD68^+$ macrophages and neutrophils, were present on the corneal endothelium after allogeneic corneal transplantation, whereas these immune cells were rarely observed in syngeneic grafts. We also demonstrated that proinflammatory cytokines, such as TNF-α and IFN-γ, were expressed by immune cells.

Methods

Ethics Statement

All animals were housed and treated in strict accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experiments were carried out under a Doshisha University Animal Care and Use Committee approved protocol (approval no. A15012), and all efforts were made to ameliorate animal suffering.

Clinical Data

The human clinical data were obtained in accordance with the tenets set forth in the Declaration of Helsinki and in accordance with a protocol approved by the Institutional Review Board of Kyoto Prefectural University of Medicine (approval no. RBMR-C-1073-1). Clinical trial registration was obtained from the University Hospital Medical Information Network (UMIN; ID: UMIN000021264) (http://www.umin.ac.jp/english/, provided in the public domain). The corneal endothelium was evaluated by contact specular microscopy (Konan scanning slit specular microscope; Konan Medical, Nishinomiya, Japan) at the follow-up clinical observations of patients who had undergone corneal transplantation at the Kyoto Prefectural University of Medicine.

Rabbit Penetrating Corneal Transplantation Model

Twenty-one eyes of 15 rabbits were grafted with either syngeneic ($n = 12$) or allogeneic ($n = 15$) corneal transplants. For syngeneic corneal transplantation, 8-mm-diameter corneal buttons were trephined (Hessburg-Barron Vacuum Trephine; Barron Precision Instruments, LLC, Grand Blanc, MI, USA), stored in media (Optisol-GS; Bausch & Lomb, Inc., Rochester, NY, USA), and transplanted back into the original eye using 10-0 nylon ophthalmic sutures (MANI Ophthalmic Sutures; MANI, Inc., Utsunomiya, Japan). For allogeneic corneal transplantation, 8-mm-diameter corneal buttons were trephined with a trephine, stored in media (Optisol-GS; Bausch & Lomb, Inc.) and transplanted into a different rabbit eye. Following corneal transplantation, 0.33-mg dexamethasone (Nichi-Iko Pharmaceutical Co., Ltd., Toyama, Japan) was injected into the subconjunctiva for 1 week. Anterior segments were assessed with a slit-lamp microscope. Corneal thickness and volume were evaluated with a camera system (Pentacam; OCULUS Optikgeräte GmbH, Wetzlar, Germany). The corneal endothelium was evaluated by contact specular microscopy (Konan scanning slit specular microscope; Konan Medical) ($n = 6$). After observation for 2 weeks, corneal specimens were evaluated by immunofluorescence stating. To analyze rejected specimens, three rabbits with transplanted allogeneic grafts were observed for up to 2 months and subjected to histologic experiments when graft rejection was observed associated with clinical symptoms such as corneal haziness, corneal edema, and conjunctival injection.

Rabbit Descemet's Stripping Endothelial Keratoplasty (DSEK) Model

For preparing DSEK graft, deep lamellar keratectomy was performed in eight rabbit corneas using microkeratome (Moria ALTK; MORIA, Inc., Doylestown, PA, USA), and the residual corneal bed was trephined with an 8-mm-diameter dermal punch (Dermapunch; Maruho Co., Ltd., Osaka, Japan). In recipient rabbits, lenses were removed using a series 20000 Legacy surgical system (Alcon, Inc., Fort Worth, TX, USA) under general anesthesia to deepen the anterior chamber 1 week prior to DSEK. Under general anesthesia, Descemet's membrane was stripped from eight eyes of eight rabbits, DSEK grafts were transplanted using the standard DSEK procedure applied in clinical settings.[1] One 0.33-mg dexamethasone dose was instilled by subconjunctival injection for 1 week. After observation for 2 weeks, corneal specimens were processed for immunofluorescence staining and scanning electron microscopy ($n = 6$). Two samples were processed for semiquantitative RT-PCR.

Immunofluorescence

Full-thickness corneal tissue was fixed in 4% paraformaldehyde for 10 minutes at room temperature, followed by perfusion in 0.5% Triton X-100 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 5 minutes at room temperature to increase tissue permeability. Nonspecific binding was blocked using 2% BSA or 2% goat serum for 1 hour at room temperature. The corneal endothelium was stained with function-related markers: ZO-1 (1:200; Zymed Laboratories, South San Francisco, CA, USA) and Na^+/K^+-ATPase (1:200; Upstate Biotechnology, Lake Placid, NY, USA). The presence of immune cells was determined using primary antibodies against CD3 (pan T lymphocyte, 1:100; Abcam, Cambridge, UK), CD4 (T-helper cells, 1:100; R&D Biotechnology, Minneapolis, MN, USA), CD8 (cytotoxic T cells, 1:100: R&D Biotechnology), CD20 (B lymphocytes, 1:100: Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), CD68 (macrophages, 1:100; Santa Cruz Biotechnology, Inc.), cathepsin G (neutrophils, 1:100; Abcam), TNF-α (1:100, Abcam), and IFN-γ (1:100, Abcam). The secondary antibody was Alexa Fluor 488-conjugated goat anti-mouse (Life Technologies, Carlsbad, CA, USA) or Alexa Fluor 594-conjugated goat anti-rabbit IgG (Life Technologies), used at a 1:1000 dilution. Cell morphology was evaluated after actin staining with a 1:400 dilution of Alexa Fluor 635-conjugated phalloidin (Life Technologies). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). The samples were observed by fluorescence microscopy (model TCS SP2 AOBS; Leica Microsystems, Wetzlar, Germany) or a confocal laser scanning electron microscopy (LEICA TCS SP8 Multiphoton; Leica Microsystems). Image J (http://imagej.nih.gov/ij/; provided in the public

TABLE. Oligonucleotide Sequences for RT-PCR

Gene	Sense Primer	Antisense Primer	Size, bp
IL-1RA	5′-GAAGTTGTGCCTGTCTTGTGTG-3′	5′-CCTCCTGGAAGTAGAACTTGGT-3′	262
IL-1b	5′-TGTTGTCTGGCACGTATGAGCTG-3′	5′-CTTCTTCTTTGGGTAACGGTTGGG-3′	227
IL-6	5′-CTGAAGAACATCCAACACCTGATC-3′	5′-CCTAACGCTCATCTTCCTAGTTTC-3′	329
IL-8	5′-ACACTCCACACCTTTCCATCC-3′	5′-CCTACGACAGATCCATGCAGT-3′	293
IL-10	5′-CCCGATCCTATTTATTTACCGAGC-3′	5′-GTTAGAAAGTGTGGTCAGGCACAG-3′	232
IL-15	5′-CTGTATCAGTGCAGGTCTTCC-3′	5′-CCTCCAGTTCCTCACATTCTTTGC-3′	310
TNF-α	5′-CTCCCAGGTTCTCTTCAGCGGTC-3′	5′-GTCCAGGTACTCAGGCTGGTTGA -3′	250
TGF-β2	5′-GATCTCCATCTACAACAGCACCAG-3′	5′-CTGAAGTAGGGTCTGTAGAAGGTG-3′	266
CX3CR	5′-TGGCCGCCAACTCCATTAAC-3′	5′-GATGGCTTTGGCTTTCTTGTGG-3′	292
CCL2	5′-CACCTGCTGCTATACATTCACC-3′	5′-GTCGTGTGTTCTTGGGTTGTG-3′	236
CCL4	5′-CAGGAGTACGTGGATGACTTG-3′	5′-CAACAGCAGAGAAACAGTGGT-3′	263
TLR4	5′-CTAACCTGTCTAGCCTTGAGCAC-3′	5′-CCACTCAGCCCTTGAATACAG-3′	240
TRAIL	5′-CTGATCCTGATCTTCACAGTGCTCC3′	5′-CTACTCTCTGAGGCCCTCTTTCTC-3′	328
GAPDH	5′-GCGTGAACCACGAGAAGTATGACAAC-3′	5′-CAGTGGAGGCAGGGATGATGTTC-3′	234

bp indicates base pairs.

domain by the National Institutes of Health, Bethesda, MD, USA) was used to create three-dimensional reconstructions.

Semiquantitative RT- PCR

Descemet's membrane, with corneal endothelium that included immune cells, was stripped from corneas of the DSEK rabbit model. Descemet's membrane stripped from healthy corneas was used as control ($n = 2$). Total RNA was isolated using a purification kit (RNeasy Mini Kit; Qiagen, Hilden, Germany). The quality of the RNA preparations was measured with a spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Ribonucleic acid was amplified using a transcriptome amplification kit (QuantiTect Whole Transcriptome Kit; Qiagen, Hilden, Germany). Specific primers, listed in the Table, were designed, and the cDNA was subjected to PCR. The internal control for gene analysis was glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Polymerase chain reactions were carried out using DNA polymerase (Extaq; Takara Bio, Inc., Otsu, Japan). The PCR amplification program consisted of denaturation at 94°C for 30 seconds, 35 cycles of annealing at 54°C for 30 seconds, and elongation at 72°C for 30 seconds. The PCR products were separated by electrophoresis on 2% agarose gels and detected under ultraviolet illumination after ethidium bromide staining.

Scanning Electron Microscopy

Corneal specimens were obtained from two syngeneic and two allogeneic PK rabbit models and two DSEK rabbit models. The specimens were washed in 0.1 M Sörensen buffer, fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M Sörensen buffer, and postfixed with 1% osmium tetroxide. This was followed by three washes in buffer before being dehydrated through an alcohol series and then transferred to hexamethyldilizane (HMDS) for two 30-minute changes (Agar Scientific, Stansted, Essex, UK). Samples were left overnight to allow the HMDS to undergo sublimation. Samples were mounted on cylinder stubs (Agar Scientific) sputter-coated with gold and examined in a JEOL 5600 scanning electron microscope (JEOL Ltd., Tokyo, Japan).

Statistical Analysis

Data are reported as means ± SEM. Differences between data sets were determined for statistical significance (*P* value) using Student's *t*-test.

RESULTS

Cell-like White Dots Were Observed on the Endothelium of Patients Following Corneal Transplantation

Representative images of a patient who underwent penetrating keratoplasty 24 years previously showed the presence of cell-like white dots on the corneal endothelium (Fig. 1A). Likewise, a patient who had undergone DSEK 5 years previously also showed the white dots (Fig. 1B). Similar white dots were

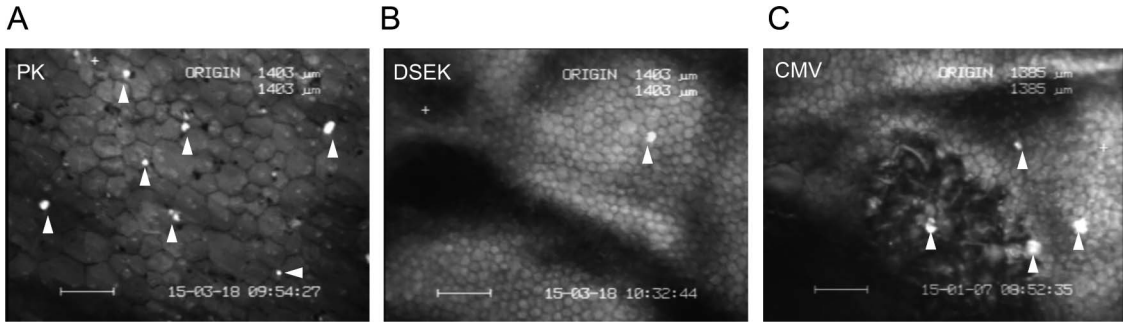

FIGURE 1. Cell-like white dots on the endothelium of patients following corneal transplantation. (**A**) Corneal endothelium was evaluated by contact specular microscopy in patients who had undergone corneal transplantation. Representative image of a 36-year-old male who underwent PK 24 years previously shows the presence of cell-like white dots on the corneal endothelium. (**B**) Image from a 67-year-old female who underwent DSEK 5 years previously, showing the cell-like white dots. (**C**) Image of a 70-year-old male who was diagnosed with CMV corneal endotheliitis infection, showing the cell-like white dots. *Arrowheads* indicate the cell-like white dots.

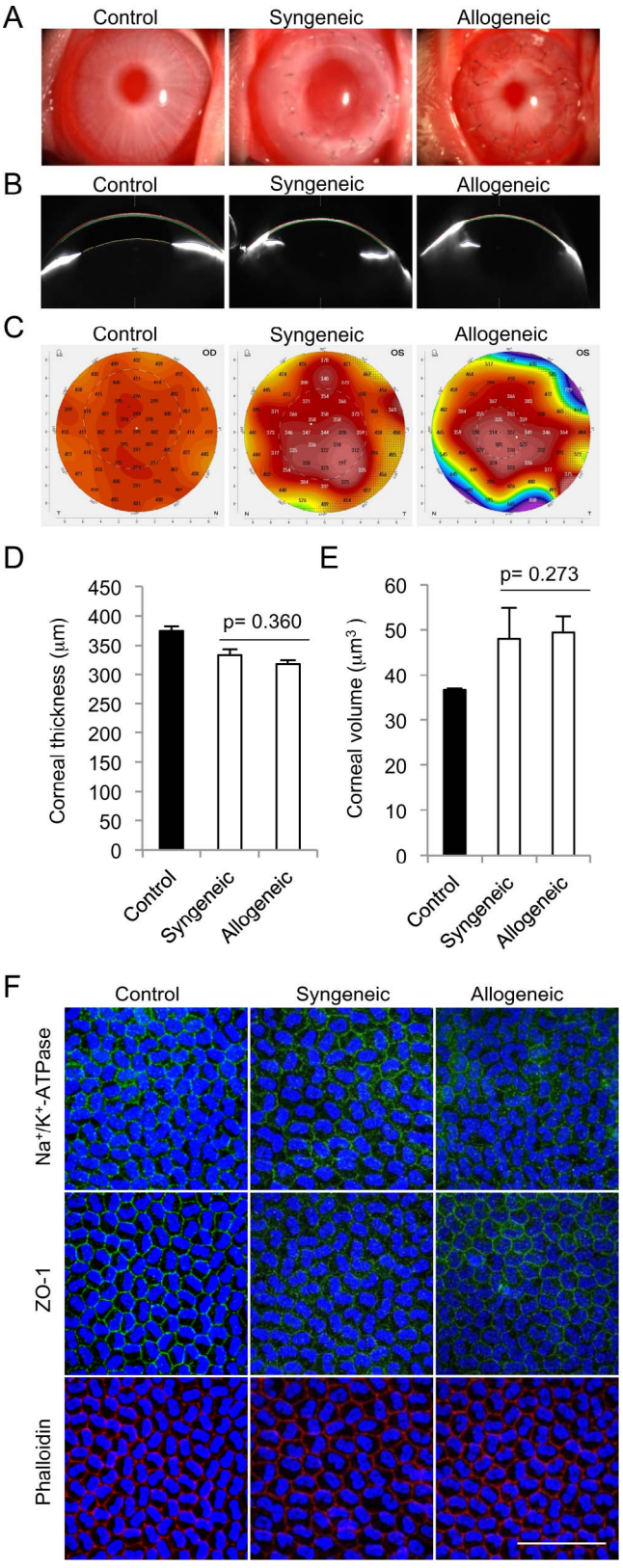

FIGURE 2. Syngeneic and allogeneic PK rabbit model. (**A**) Representative anterior segment images (*right*: control healthy eye; *middle*: syngeneic PK; and *left*: allogeneic PK) obtained by slit-lamp microscopy 14 days after transplantation are shown. (**B**, **C**) Anterior segment was also evaluated. Representative images and corneal thickness maps are shown. Values of the corneal thickness and corneal volume maps are indicated in micrometers. (**D**, **E**) Central corneal thickness and corneal volume were analyzed. No significant difference was noted in the central corneal thickness and corneal volume between the syngeneic and allogeneic transplantation groups. (**F**) Corneal endothelial structure and function of the syngeneic and allogeneic grafts were examined histologically 14 days after transplantation. Corneal endothelial morphology was assessed by actin staining, and endothelial function was assessed by immunostaining for ZO-1 and Na^+/K^+-ATPase. Cell nuclei were stained with DAPI. Representative images from at least two independent experiments are shown. *Scale bar*: 100 μm.

observed on the corneal endothelium of a patient who was diagnosed with cytomegalovirus (CMV) corneal endotheliitis, which is an inflammatory disease due to CMV,[13,14] suggesting that the white dots were immune cells recruited due to an immune response (Fig. 1C).

Allogeneic Corneal Transplantation Recruits Immune Cells in Rabbit Model

We used a rabbit PK model to determine whether the white dots observed on corneal endothelium were immune cells. Slit-lamp microscopy showed that a transparent corneal graft was maintained at 2 weeks postoperatively in both syngeneic and allogeneic groups (Fig. 2A). No clinical features of corneal allograft rejection, such as endothelial keratic precipitates, rejection lines, or severe conjunctival injection were observed in allogeneic or syngeneic grafts during the observation time of 2 weeks. Scheimpflug images and corneal thickness maps obtained with a Pentacam high-resolution camera also showed that an anatomically normal cornea had been restored by corneal transplantation (Figs. 2B, 2C). No significant difference was noted in the central corneal thickness and corneal volume between the syngeneic and allogeneic groups (Figs. 2D, 2E). The endothelial phenotype of the CECs in the syngeneic and allogeneic corneal grafts was retained, along with expression of function-related markers, such as ZO-1 (marker of barrier function) and Na^+/K^+-ATPase (marker of pump function), and the hexagonal monolayer morphology (Fig. 2F). Contact specular microscopy examination showed healthy morphology of corneal endothelium in both syngeneic and allogeneic groups 14 days after corneal transplantation. However, cell-like white dots that were similar to the dots observed in human subjects following corneal transplantation were observed on corneal endothelium in the allogeneic group (Fig. 3A). Scanning electron microscope analysis revealed the normal hexagonal morphology of the corneal endothelium in both the graft and peripheral recipient cornea in the syngeneic model as well as in control corneas. However, scanning electron microscope analysis revealed the presence of numerous cells that were well distinguished from the corneal endothelium on the surface of the allogeneic graft endothelium, and to a significantly lesser extent, on the recipient corneal endothelium. Notably, very few of these cells were observed in the syngeneic group, but massive numbers of cells were observed in the allogeneic group. Cell–cell junctions were partially disrupted, and membrane blebbing, presumably associated with the apoptotic process, was observed in nearby cells in the corneal endothelium (Fig. 3B). Taken together, these findings supported our hypothesis that the cell-like white dots were immune cells that might be exerting potentially deleterious effects on the corneal endothelium.

T-Helper Cells, Cytotoxic T Cells, B Lymphocytes, Macrophages, and Neutrophils Infiltrate the Corneal Endothelium of Allogeneic Corneal Grafts

We conducted an immunohistochemical analysis to characterize the infiltrated cells present on the corneal endothelium

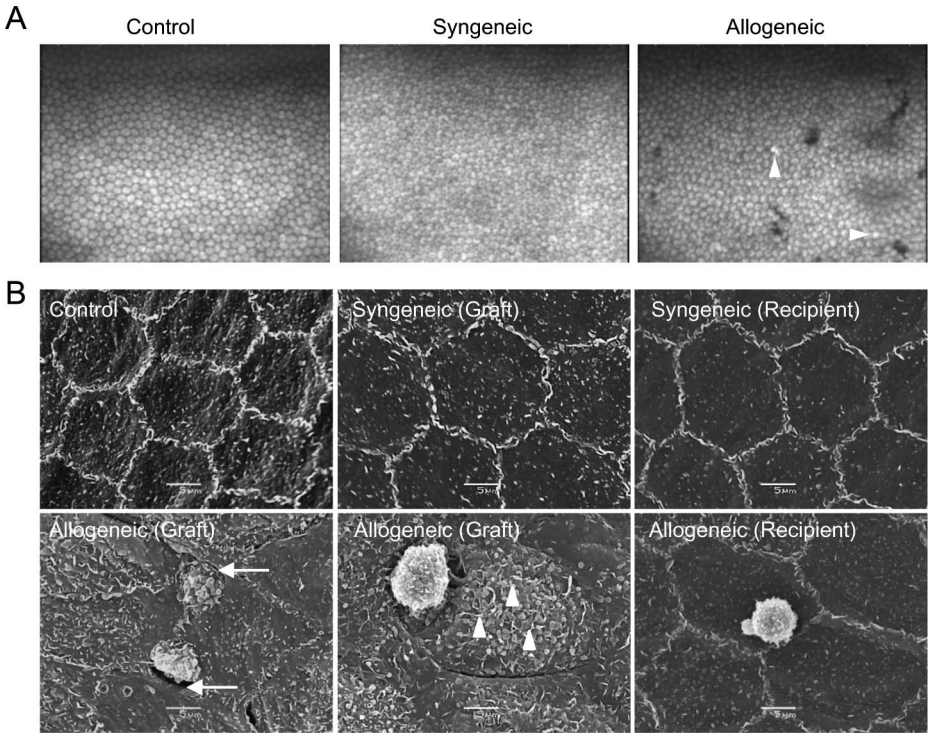

Figure 3. Contact specular and scanning electron microscopy analysis of the corneal endothelium in the syngeneic and allogeneic corneal grafts. (**A**) Corneal endothelial cell morphology was also assessed by contact specular microscopy, and representative images are shown for each experimental group. In the allogeneic PK group, the corneal graft endothelium shows cell-like white dots, indicated by *white arrowheads*. (**B**) Scanning electron microscope images of the corneal endothelium of a control healthy cornea, a syngeneic graft, and an allogeneic graft. Cell–cell junctions were partially disrupted (*arrows*), and membrane blebbing (*arrowheads*) was observed near the immune cell–like cells on the corneal endothelium of the allogeneic graft. All images are representative of two animals in each group. *Scale bars*: 5 μm.

after allogeneic corneal transplantation in rabbits. None of the grafts (syngeneic or allogeneic) displayed any clinical signs of organ rejection, but immune cells, characterized by the presence of $CD4^+$ T-helper cells, $CD8^+$ cytotoxic T cells, $CD20^+$ B lymphocytes, $CD68^+$ macrophages, and neutrophils marked by cathepsin G, were observed on the endothelial layer in nine out of nine allogeneic graft specimens 2 weeks after transplantation. Similar to the scanning electron microscope findings, these immune cells were rarely observed in the syngeneic grafts (Fig. 4A). As a positive control, where infiltration of immune cells would be expected, we analyzed corneal grafts that had been rejected 2 months after allogeneic transplantation. All three rabbits that had been observed for 2 months exhibited signs associated with graft rejection, such as corneal haziness, corneal edema, and conjunctival injection at 1.5 to 2 months. The rejected corneal grafts exhibited distinct morphologic alterations as determined by the cellular distribution of F-actin, which suggested CEC transformation into fibroblast-like cells, as occurs in human corneal graft rejection. Higher numbers of infiltrating immune cells—$CD4^+$ T-helper cells, $CD8^+$ cytotoxic T cells, $CD20^+$ B lymphocytes, $CD68^+$ macrophages, and neutrophils—were observed on the rejected corneal endothelium than on the nonrejected allogeneic graft (Fig. 4A).

Immune cells secrete cytokines that are critical regulators of immune response; therefore, we investigated whether proinflammatory cytokines were secreted in corneal grafts. Tumor necrosis factor-α and IFN-γ were detectable in the allogeneic graft endothelium, which showed no clinical features of rejection 2 weeks after corneal transplantation, but TNF-α and IFN-γ were not detected in the syngeneic graft or the control (Fig. 4B). Co-staining showed that a certain population of $CD4^+$ T-helper cells expressed TNF-α and IFN-γ and that a certain population of $CD8^+$ cytotoxic T cells expressed TNF-α and IFN-γ in the allogeneic grafts 2 weeks after surgery (Fig. 4C).

Immune Cells Infiltrate on the Corneal Endothelium of Allogeneic DSEK Graft

We next determined the infiltration of immune cells onto the corneal endothelium of a DSEK graft, as the CEC declines seen after transplantation of DSEK grafts are at the same level (or even worse) as that seen in PK.[6,15–17] Corneal transplantation performed as a DSEK procedure in the rabbit gave a successful graft in all cases, with a transparent cornea and no serious complications such as graft rejection, graft failure, or graft dislocation. Representative slit-lamp microscopic images showed attachment of the graft to the back side of cornea, and corneal transparency was maintained in the rabbit model 2 weeks after surgery (Fig. 5A). Scanning electron microscope demonstrated the presence of numerous discrete nonendothelial cells, morphologically similar to immune cells in appearance, present near the graft–recipient interface (Fig. 5B). Notably, a high density of immune-like cells was observed on the corneal endothelium of the DSEK graft in contrast to the recipient corneal endothelium, where very few immune-like cells were found (Fig. 5C). The control eyes exhibited the normal hexagonal morphology of the corneal endothelium without any immune-like cells. Higher magnification scanning electron microscope images showed that immune-like cells were adjacent to a missing endothelial cell (Fig. 5D), suggesting that these cells may have the ability to damage the corneal endothelium.

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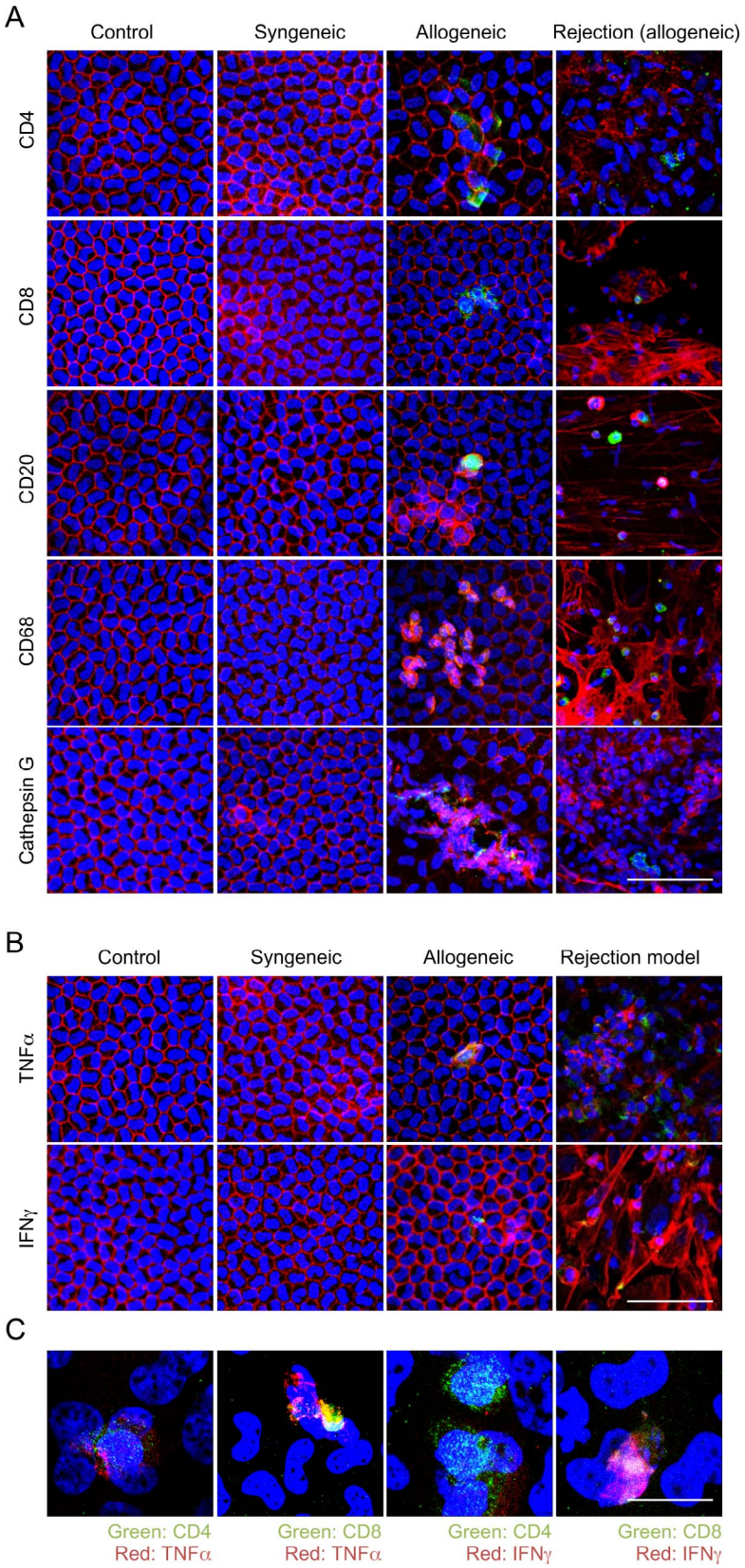

FIGURE 4. Immunofluorescence analysis of the corneal endothelium of the syngeneic and allogeneic PK model. (**A**) Control cornea, syngeneic corneal graft, allogeneic corneal graft, and rejected allogeneic corneal graft were immunostained with CD4 (T-helper cells), CD8 (cytotoxic T cells), CD20 (B cells), CD68 (macrophages), and cathepsin G (neutrophils) antibodies 14 days post transplantation. $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, $CD20^+$ B lymphocytes, $CD68^+$ macrophages, and neutrophils characterized the allogeneic PK graft endothelium layer and the rejected graft cornea. DAPI was used to stain cell nuclei. *Scale bar*: 100 μm. (**B**) Secretion of TNF-α and IFN-γ was determined in the control corneal

syngeneic corneal graft, allogeneic corneal graft, and rejected allogeneic corneal graft. Actin and cell nuclei were stained with generic term and DAPI, respectively. All experiments were performed in duplicate. *Scale bar*: 100 μm. (**C**) Co-staining of CD4/TNF-α and IFN-γ, and CD8/TNF-α and IFN-γ in allogeneic grafts 2 weeks after corneal transplantation. Nuclei were stained with DAPI. Experiments were performed on three independent animals. *Scale bar*: 50 μm.

Similar to the PK model, immunofluorescence analysis demonstrated the presence of $CD3^+$ T lymphocytes, $CD4^+$ T-helper cells, $CD8^+$ cytotoxic T cells, $CD20^+$ B lymphocytes, and $CD68^+$ macrophages on the corneal endothelium (Fig. 5E). The recipient endothelium rarely showed the presence of these immune cells, suggesting that the allogeneic corneal endothelium has the ability to recruit immune cells. Consistent with the findings observed previously for the PK corneal grafts, TNF-α– and IFN-γ–producing immune cells were also detected in the DSEK graft endothelium (Fig. 5E). Image stacks of specific depths and high resolution obtained by confocal laser scanning electron microscopy of the DSEK corneas revealed that these immune cells were attached onto the graft endothelium layer positioned at the anterior chamber site (Fig. 5F). The expression of cytokines related to inflammation was evaluated 2 weeks after DSEK in corneal endothelium that included immune cells. Semiquantitative RT-PCR showed higher expression of IL-1 receptor antagonist (IL-1RA), IL-1b, IL-6, IL-15, TNF-α, TGF-β2, CX3CR, and CCL2 in DSEK corneal endothelium than in healthy corneal endothelium, while IL-8, IL-10, TLR4, and TRAIL were detected at similar levels in both groups (Fig. 5G).

Discussion

The cornea is an immune-privileged tissue with properties that prevent alloimmune-mediated rejection responses, which then enables an advantageously high success rate of transplantation.[18–24] Numerous basic research studies have demonstrated that the immune privilege of the eye is not solely a phenomenon due to immunologic ignorance, but instead is a highly orchestrated immune system consisting of unique immune suppression mechanisms, widely known as anterior chamber–associated immune deviation.[21–23,25] This immune-privileged environment, as well as the underlying mechanisms involved in corneal graft rejection by host immune systems, has also been intensively investigated.[22,26–29]

By contrast, the mechanism of late endothelial failure is still unclear. The clinical data showing the much lower drop in cell density of CECs after autologous rotational corneal transplantation than after allogeneic transplantation suggested the possibility that immunologic influences are involved in the pathogenesis of chronic endothelial failure.[12] We showed that cell-like white dots can be observed on the corneal endothelium of a transplanted graft by scanning slit contact specular microscopy in the patients who show no clinical features of graft rejection. Similar cell-like white dots were also observed on the corneal endothelium of the CMV corneal endotheliitis patients, so we speculated that these dots might be immune cells. In agreement with our findings, in vivo confocal microscopy revealed the presence of indistinguishable small, rounded white cell bodies representing inflammatory cell structures on the corneal endothelium following corneal transplantation.[30,31] Our rabbit model experiments demonstrated that the cell-like white dots observed by scanning slit contact specular microscopy correspond to immune cells comprising at least T-helper cells, cytotoxic T cells, B cells, macrophages, and neutrophils. One limitation of our study is that these cell-like white dots, revealed to be immune cells, were observed 2 weeks after surgery in the rabbit model, but they were observed in humans a longer time after the corneal transplantation. Further investigation, such as using postmortem corneal specimens in the patients, should determine whether immune cells exist on the corneal endothelium of patients who do not exhibit clinical features of graft rejection. Another important question to be answered is whether this immunologic response is induced owing to the surgical intervention or to the allogeneic graft, as leukocyte extravasation and migration to injury sites are important milestones of wound-healing process. Our histologic assessment demonstrated that immune cells were rarely observed on syngeneic grafts, whereas they were intensively observed on allogeneic grafts. This suggests that immune cells were recruited by allogeneic grafts, though the nature of the signal—whether immune cells recognized CECs or corneal stromal cells or did not recognize allogeneic cells—should be further investigated. In agreement with previous reports,[26–29] rejected allogeneic grafts were characterized by inflammatory cell aggregates composed of $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, macrophages, and neutrophils and by tissue destruction. Interestingly, unlike the case for the rejected grafts, the cells observed in transparent allogeneic grafts without clinical features of rejection were not associated with destruction of the surrounding CECs. One possible explanation for the presence of immune cells on a nonrejected graft is that they have a potency to precede typical graft rejection. Though this is speculative, if the grafted cornea successfully evaded rejection triggered by these immune cells, the graft would eventually exhibit CEC loss. Graft rejection is typically observed after 2 months; therefore, analysis of animal models for a longer time will help in understanding the role of these immune cells. Another possible explanation is that the corneal endothelium was damaged by an as yet unknown mechanism, and the immune cells were recruited for a wound-healing process. Further functional experiments to evaluate the cytotoxicity of the adhered immune cells to the graft corneal endothelium are needed to verify the role played by immune cells in corneal endothelial damage.

Penetrating keratoplasty was previously the only procedure for corneal transplantation, but new procedures, such as DSEK and DMEK (Descemet's membrane endothelial keratoplasty), have been developed for replacement of the corneal endothelium that do not involve full-thickness replacement.[1] Stripping endothelial keratoplasty and DMEK are less-invasive treatments and are now frequently performed.[32,33] Anshu et al.[34] reported rejection rates of 17% in PK, 9% in DSEK, and 0.7% in DMEK in a single center and suggested that DSEK and DMEK reduced the risk of rejection. This may be because DSEK and DMEK grafts do not include epithelium, as each layer of the cornea has different immunogenicity. Indeed, the use of reconstituted mouse corneal grafts confirmed that the corneal epithelium had a higher allosensitization potency when compared with endothelium.[35–37] By contrast, the incidence of graft rejection in DSEK, and even in DMEK, suggests that the corneal endothelium also has immunogenicity. Our current data for our rabbit DSEK model showed that immune cells migrated onto allogeneic graft corneal endothelium and expressed proinflammatory cytokines, as observed in PK. We also showed that the levels of cytokines related to inflammation are higher in the DSEK corneal endothelium. However, the source of these cytokines (i.e., the corneal endothelium or the

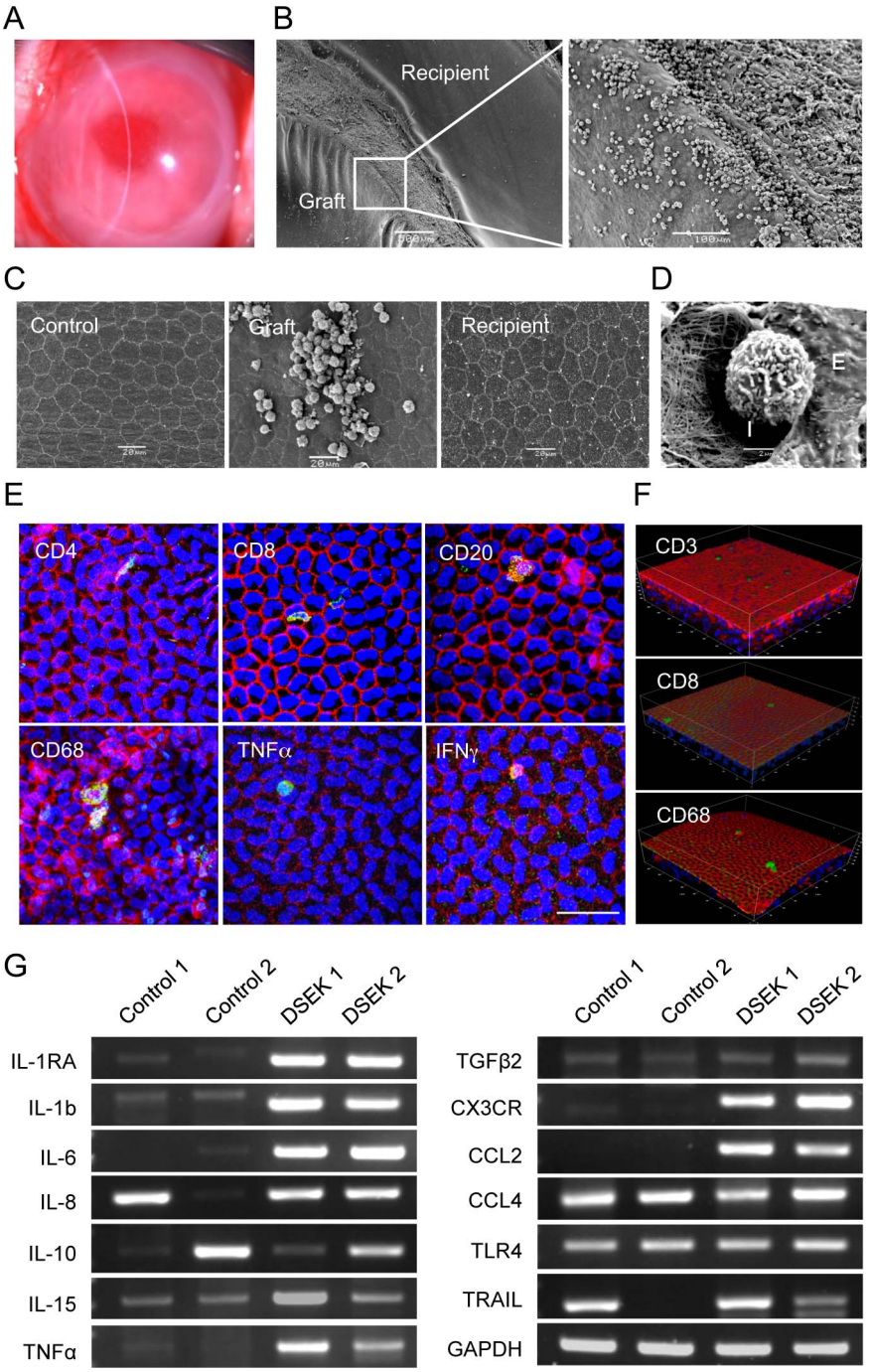

Figure 5. Involvement of immune cells in the corneal endothelium of a DSEK graft. (**A**) Corneal transplantation was performed as a DSEK procedure in six rabbits, and a representative slit-lamp microscopy image is shown. (**B**) Scanning electron microscopy evaluation of the donor–host junction of the DSEK rabbit model. *Scale bar*: 500 μm. Higher magnification of the boxed area shown on the right-hand side. *Scale bar*: 100 μm. (**C**) Scanning electron microscope demonstrated that numerous nonendothelial cells, morphologically with an immune cell–like appearance, were present near the graft–recipient interface. A high density of these immune-like cells was observed on the corneal endothelium of the DSEK graft, whereas the recipient corneal endothelium showed very few immune-like cells. The control eyes exhibited a hexagonal, normal morphologic corneal endothelium without any immune-like cells. All images are representative of two animals in each group. *Scale bar*: 2 μm. (**D**) Higher magnification scanning electron microscope image showing immune-like cells adjacent to a missing endothelial cell. *Scale bar*: 2 μm. (**E**) Corneal endothelium of the DSEK graft immunostained with immune cell–related markers: $CD4^+$ cell (T-helper cells), $CD8^+$ cell (cytotoxic T cells), $CD20^+$ cell (B cells), and $CD68^+$ cell (macrophages). Production of TNF-α and IFN-γ was also evaluated by immunostaining. Actin and cell nuclei were stained with Alexa Fluor 594–conjugated phalloidin and DAPI, respectively All experiments were performed in triplicate. *Scale bar*: 100 μm. (**F**) Stripping endothelial keratoplasty corneas were analyzed by confocal laser scanning electron microscopy. Image stacks up to a depth of 150 μm, at either 1- or 3-μm intervals, were acquired, and three-dimensional reconstructions were created. Representative still images for $CD3^+$ T lymphocytes, $CD8^+$ T lymphocytes, and $CD68^+$ macrophages on the corneal endothelium are shown. Actin staining was performed to evaluate cell morphology. Nuclei were stained with DAPI. (**G**) The expression of cytokines related to inflammation was evaluated 2 weeks after DSEK in corneal endothelium that included immune cells by semiquantitative RT-PCR.

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immune cells) was not established in the current study. Nevertheless, the elevated cytokine levels might suggest an involvement of immune cells as part of the underlying mechanism of CEC loss in corneal endothelial transplantation performed using procedures other than just conventional PK.

In conclusion, our data suggest that T lymphocytes, B lymphocytes, macrophages, and neutrophils are present on the grafted corneal endothelium following both PK and DSEK. The current findings should stimulate further research aimed at clearly elucidating the involvement of immune cells as an underlying pathophysiology to provide treatment for late endothelial failure.

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