·Basic Research·

Lymphocyte infiltration and activation in iris-ciliary body and anterior chamber of mice in corneal allograft rejection

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Abstract

• AIM: To investigate the infiltration and activation of lymphocyte in iris-ciliary body and anterior chamber after allogenic penetrating keratoplasty (PK), for further revealing the role of iris-ciliary body in corneal allograft immune rejection.

• METHODS: In the mice models of PK, BALB/C mice received orthotopic isografts (n=35) or C57BL/6 donor allografts (n=25). Grafts were examined daily for 3 weeks by slit-lamp microscopy and scored for opacity. The infiltration of $CD4^+$ T lymphocyte in iris-ciliary body and anterior chamber was examined by immunohistology and the mRNA of CD80 and CD86 in both cornea graft and iris-ciliary body by RT-PCR was analyzed in allograft recipient at days 3, 6, 10 and the day when graft rejection occurred. Isograft recipients were examined as control at the corresponding time points. Transmission electron microscope was used to study the ultrastructure, especially cell infiltration, of iris-cilary body and corneal graft at day 3, 7 and the day when rejection occurred after allogenic PK.

• RESULTS: Rejection was observed in all the allograft recipients followed more than 10 days, at a median time of 15 days (range 12-18 days), but not in any of isografts. $CD4^+$ T cells were first detected at day 6 after transplantation in limbus and Ciliary body, and then in the stroma of recipient, iris, anterior chamber and corneal allograft with an increased number until graft rejection occurred. CD80 and CD86 mRNA were detected under RT-PCR examination in both graft and iris-ciliary body of allograft recipient, but not in any of isograft recipient. Three days after operation, lymphocytes and monocytes macrophages were visible in iris blood vessels and the anterior chamber, and vascular endothelial cell proliferation and activation were significant under transmission electron microscopy examination. At day 7, corneal endothelial cells became thinner. Lymphocytes and mononuclear macrophages were found with great number in the anterior chamber and adhered to the corneal endothelium. Blood vessels in iris increased and were filled with lymphocytes. And lymphocytes were detected to migrate through endothelial cell gap out of vessels. When allograft rejection occurred, macrophages attached to endothelial cells with large number of lymphocytes and macrophages infiltrating in iris.

• CONCLUSION: Lymphocyte infiltration and activation occurred in iris-ciliary body after allogenic PK, and the lymphocytes could migrate from iris blood vessel to the anterior chamber, which might play an important role in corneal allograft immune rejection.

• KEYWORDS: lymphocyte; corneal transplantation; penetrating; graft rejection; iris-ciliary body; anterior chamber
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INTRODUCTION

Allograft rejection is a leading cause of corneal graft failure and thus a crucial indication for repeating keratoplasty [1,2]. Investigation of the mechanism and treatment of allograft rejection are the central goals of much

of the current researches. The immune response has three fundamental, sequential steps: 1) the inductive stage in which alloantigens are delivered to a regional lymphoid tissue by either donor 'passenger' antigen presenting cells (direct pathway) or by host antigen presenting cells (indirect pathway); 2) antigen presentation and activation of alloreactive T cells in the regional lymph nodes; 3) expression of allodestructive mechanisms within the graft. Conventionally, limbus blood and lymphatic vessels were considered as the only conduits for egression of alloantigens to the peripheral immune apparatus and migration of circulating effector immune elements into the corneal allograft. But it is not reasonable enough to explain corneal graft rejection after penetrating keratoplasty (PK). In the cases of PK, endothelial rejection other than stromal and epithelial rejection is the leading cause of graft failure and is observed in approximately 20% of the recipients of PK [3,4].

The anterior chamber was widely noted in corneal graft rejection because of anterior chamber-associated immune deviation (ACAID), which was considered as one of the fundamental adaptations that provide corneal allograft with immune privilege. Orthotopic corneal allografts are in direct contact with the anterior chamber and it has been suggested that corneal antigens are sloughed into the anterior chamber during corneal transplantation and induce ACAID [5]. However, immune privilege of corneal allografts is abolished in virtually any condition in which inflammation, neovascularization, or trauma is elicited in the cornea, and most keratoplasty patients have corneal graft beds that have lost their immune privilege due to preceding corneal inflammation, neovascularization, trauma, or infections [1,6]. In human corneal graft rejection, inflammatory cells aggregating on the endothelium in many allograft recipients are visible on microscopy *in vivo*. Also, immune cells were detected in the anterior chamber of patients and animal models with immune reactions after PK [7,8]. Hence, it was assumed that immune cells reached a corneal allograft by two routes, from vessels in the peripheral recipient cornea, and from vessels in the recipient iris *via* the aqueous humour [8]. But iris-ciliary body has received little attention in previous studies of experimental corneal graft rejection.

The present study is designed to detect the infiltration and activation of $CD4^+$ T lymphocytes in iris-ciliary body and anterior chamber during the immune rejection in a mice PK model. It aims to further confirm the effect of anterior chamber pathway in the corneal graft rejection and to provide more adequate theoretical basis for revealing the mechanism of corneal immune rejection.

MATERIALS AND METHODS

Materials Five to 8 weeks old female BALB/c and C57BL/6 mice were purchased from Beijing Experimental Animal Center. Mice were housed in a specific pathogen-free environment at the Shandong Eye Institute animal facility. All animals were treated according to guidelines established by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Public Health Policy on Humane Care and Use of Laboratory Animals (US Public Health Review), and all procedures were approved by the Institutional Animal Care and Use Committee.

Methods

Corneal transplantation Before transplantation, cyclopentolate 1% drops were administered to graft recipient eyes to dilate the pupil. Anesthesia was administered intraperitoneally by ketamine/xylazine solution at a dose of 120mg/kg body weight and 20mg/kg body weight respectively. A 2.0mm diameter full thickness corneal disc was trephined from the centre of the donor cornea and grafted into the centre of the recipient cornea [9,10]. After application of antibiotic ointment, the eyelids were closed for 3 days.

Examination of grafts Using a slit-lamp microscopy, recipient corneas were examined daily following operation for up to 21 days. Mice with surgical complications (cataract, anterior synechiae or failure to form an anterior chamber by day 2) which might either prejudice graft outcome or confuse possible diagnosis of graft rejection were excluded from the study. Graft appearance was assessed and the opacity graded according to the following scale modified for pigmented iris: 0-no opacity; 1-minimal-all iris details (crypts) visible; 2-some iris details visible; 3-strongonly pupil margin visible; 4-complete-anterior chamber not visible. An opacity $\geqslant 3$ was considered rejected.

Experimental groups BALB/c mice were used as recipients and syngeneic graft donors. Allograft donors were C57BL/6 mice.

Immunohistology Mice (n=3 per group) were sacrificed at several time points after surgery (days 3, 6, 10, and the day when graft rejection occurred in allograft recipient and the corresponding time point in isograft recipient) by overdose chlorine ketamine anesthesia. Both eyes were enucleated and postfixed in gel. Cryostat sections were cut at 5μm thickness and mounted on gelatin-coated slides. Following fixation of sections in acetone for 10 minutes, endogenous peroxidase activity was blocked with 0.3% protamine for 20 minutes. Sections were then incubated with 1:50 dilution of rats anti-mouse CD4 IgG (purchased from the United States the BD Phamingen company) for 120 minutes, washed in 0.02MPBS, incubated with 1:100 fluorescein isothiocyanate (FITC) labeled goat anti-rat monoclonal antibody (purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 30 minutes, washed in MPBS and mounted. One section through the center (on account of the circular graft shape, this could be identified by the maximum diameter on sequential sections) of each graft specimen was examined by fluorescence microscopy. To describe the degrees of $CD4^+$ T lymphocyte infiltration, three adjacent fields were examined at ×40 and the mean calculated. Antibody labelling of cells infiltrating graft

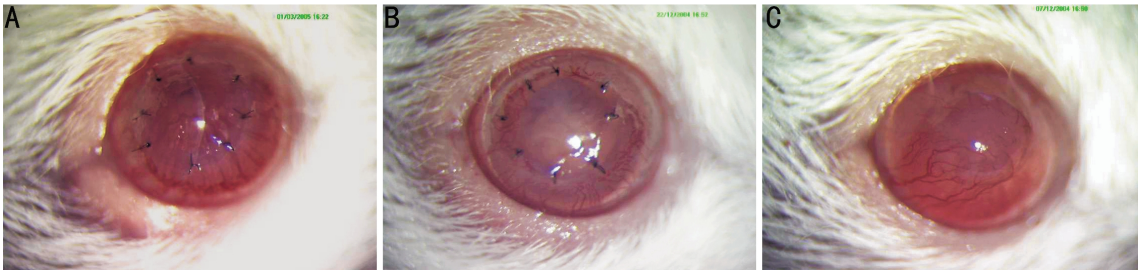

Figure 1 BALB/c mice that received corneal allograft transplantation from C57BL/6 mice.

epithelium, the iris-ciliary body and anterior chamber was scored semiquantitatively on an arbitrary scale of - to +++ (Table 1).

Rt-PCR Every 3 mice with allografts and isografts were sacrificed at the time points just as that in immunohistology examination. The eyes were enucleated, and total RNA isolation from corneal grafts and iris-ciliary body was carried out using the EZ-10 spins Colunm RNA isolation Kit (Bio Basic Inc. Canada). RT-PCR was performed using the Qiagen One Step RT-PCR Kit (Qiagen, Courtaboeuf, France) which provides enzymes for both the reverse transcription and the PCR. The first-strand cDNA was synthesized after incubation at 50℃ for 30 minutes and PCR conditions were 95℃ for 15 minutes followed by 35 cycles of 94℃ for 30 seconds, 60℃ for 30 seconds, 72℃ for 1 minutes, and finally at 72℃ for 10 minutes. The sequences of the primers for mice CD80 and CD86 (synthesized by Shanghai bio-genetic company) are presented in Table 2. The amplified products were separated using 2% agarose gel electrophoresis stained with ethidium bromide.

Electron microscopy Ultrastructural analysis of the iris-ciliary body and corneal graft was performed by transmission electron microscopy. Every 3 mice with allografts were sacrificed on days 3, 7 and the day when graft rejection occurred. The eyes were enucleated, and the globes were carefully incised (1-mm cut) immediately behind the limbus, by using a sharp razor blade. Curved iris scissors were then used to continue the incision around the globe and separate the eye into anterior and posterior segments. Three or four radial incisions were made with the razor blade through the anterior segment to produce pie-shaped wedges consisting of cornea-sclera externally and iris-ciliary body internally. Three healthy eyes from BALB/c mice were processed accordingly and served as control. All the samples were postfixed in 2% buffered osmium tetroxide, dehydrated in graded alcohol concentrations, and embedded in epoxy resin (Epon 812; Fluka, Buchs, Germany) according to standard protocols. Thin and ultrathin sections were cut and stained with uranyl acetate and lead nitrate to visualize the morphology of cornea, iris-ciliary body with transmission electron microscope.

RESULTS

Clinical Results A total 35 allografts and 25 syngeneic grafts were successfully performed. Two cases in allografts group and one case in syngeneic grafts group developed cataract one week after surgery, which were excluded from experimental group and replaced with supplementary cases. Three days after operation, angiectasis was noted on limbus in both groups under slit lamp microscope examination (Figure 1A). New blood vessels were seen around the sutures at day 5 to 6 after operation, and then advanced to the corneal graft along with graft edema at day 7 to 9 (Figure 1B). After suture removal at day 9, neovascularization in syngeneic grafts disappeared and the graft returned clarity within one week. But in allografts group, neovascularization was further aggravated and rejection occurred at a median of 15 days (range 12-18 days, Figure 1C).

Table 1 Grade scale of $CD4^+$ T lymphocytes infiltration

Grade	$CD4^+$ T lymphocytes (*n*, [1]cells/HPF)
-	0
+	1-10
++	11-20
+++	21-30
++++	⩾30

[1]high power field.

Table 2 Primers selected for pcr testing after reverse transcription

Gene	Sequence	PCR product size (bp)
CD80	F,5-GGTATTGCTGCCTTGCCGTT-3 R,5-TCCTCTGACACGTGAGCATC-3	546
CD86	F,5-TCCTGTAGACGTGTTCCAGA-3 R,5-TGCTTAGACGTGCAGGTCAA-3	521

Immunohistochemistry At day 3 after operation, the earliest interval examined post-graft, no $CD4^+$ T lymphocytes were detected in both groups (Figure 2A). Higher numbers of infiltrating inflammatory cells were seen in all allografts than that in isografts at all following time points. At day 6, isografts contained few inflammatory cells (+) other than at the wound suture. In most allografts, $CD4^+$ T lymphocytes were first detected with small number (+) in corneal limbal stroma and ciliary body (Figure 2B). After suture removal at day 9, infiltrating T lymphocytes still aggravated and accumulated into a cell lake (+++) at the angle of anterior chamber. Also, it was detected in iris and anterior chamber (++). In most allografts, $CD4^+$ T lymphocytes were seen within the endothelium as well as the peripheral stroma of graft (Figure 2C). Increased numbers of infiltrating T lymphocytes (++++) in allografts

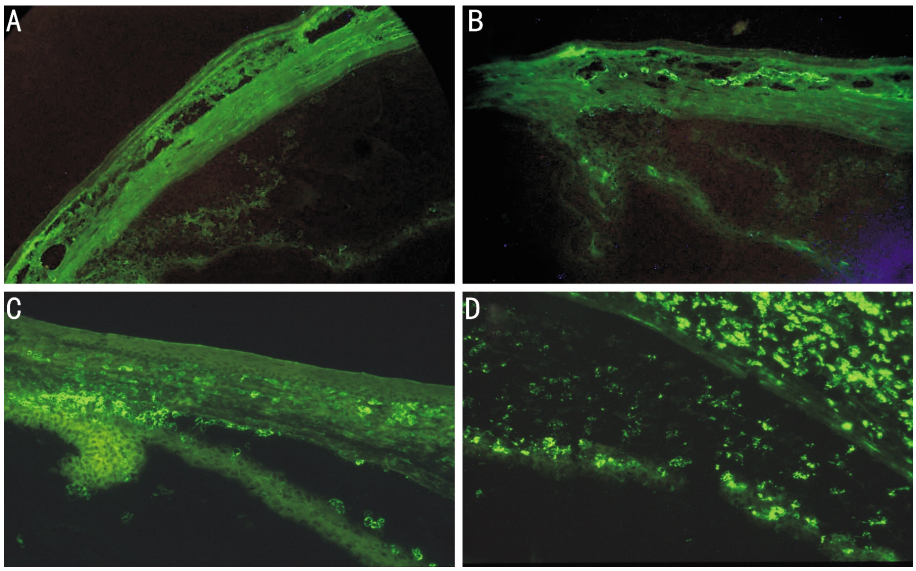

Figure 2 CD4⁺ T lymphocytes infiltration in iris-ciliary body, anterior chamber and graft by immunohistochemistry during the process of corneal allograft rejection.

and anterior chamber were found when immune rejection occurred (Figure 2D). In isografts, CD4⁺ T lymphocytes could be detected still in small number (+) and diminished following suture removal and transparency recovery of grafts.

Cytokine Expression Under RT-PCR examination, CD80 and CD86 mRNA were detected in both cornea graft and iris-ciliary body in allograft recipient at all time points after transplantation. While in isograft recipient, not any of the cytokines mentioned above were detected at any time point (Figure 3).

Ultra Microstructure Under the transmission electron microscope, the structures of cornea, limbus, and iris-ciliary body were clear, and the descemet membrane and endothelial cell layer were integrated. No inflammatory cells were detected in any of the structures (Figure 4A).

Three days after surgery, the arrangement of keratocyte and collagen fibers began turn into disorder. Dense nucleus chromatin was found in keratocyte (Figure 4B↖) and endothelium (Figure 4B↘), and the nucleoli became prominent. In the corneal endothelial cells, the nucleus got bigger in size with incisura, the number of mitochondria increased, and the nucleoli became irregular and increased in size (Figure 4B↘). The iris, especially in the surface layer, showed a strong inflammatory reaction. Inflammatory cells, mainly lymphocytes and mononuclear macrophages, were found in the blood vessels of iris (Figure 4B↙). In addition, vascular endothelial cell proliferation, and activation was significant, with increased nucleolus and dense chromatin (Figure 4B↗). Mononuclear macrophages could be found in the anterior chamber (Figure 4B→). No inflammatory cells were detected in anterior chamber angle, but the nucleolus got larger, chromatin became dense, and cell arrangement turn into disorder.

At day 7, corneal parenchymal cells showed active

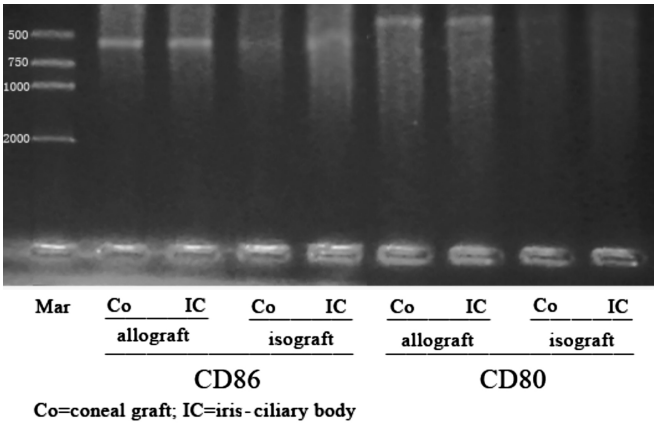

Figure 3 RT-PCR analysis of CD80 and CD86 expression in cornea graft and iris-ciliary body after transplantation.

proliferation, including dense nuclear chromatin, large nucleus and increased mitochondria (Figure 4C↘). The corneal endothelium turned thinner (Figure 4C↖). Lymphocytes (Figure 4C↗) and mononuclear macrophages (Figure 4C→) were found with great number in the anterior chamber, and some of the cells adhered to the corneal endothelium. The iris got obvious edema and thickening. The blood vessel in iris increased in number, and was filled with large amount of lymphocytes (Figure 4D←), which were found attached to the inner wall in part. Also, lymphocytes were found emigrate through the endothelial cell gaps (Figure 4D↗).

When corneal graft rejection occurred, the inflammatory response in corneal graft got further aggravated. Keratoc

ytes in stroma showed active proliferation, with a large number of pseudopodia emerged on cell surface, and the nuclei were significantly enlarged (Figure 4E↙). Corneal endothelium was attached by the monocyte-macrophage cells (Figure 4E↖). The thickness of iris was further increased, especially the posterior layer, along with infiltration of a

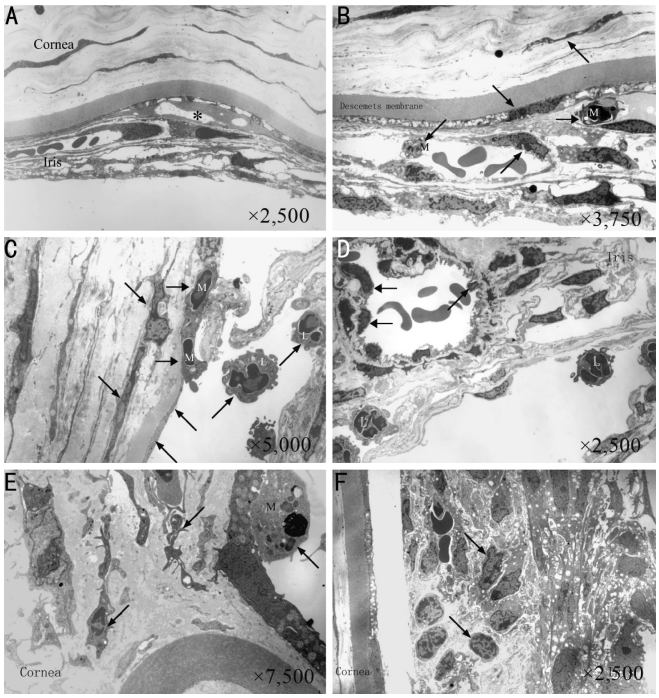

Figure 4 Ultra microstructure of iris-ciliary body and corneal graft during the process of corneal allograft rejection.

large number of inflammatory cell with low electron density, nuclear chromatin margination and condensation (Figure 4F↘).

DISCUSSION

The iris-ciliary body, with the most abundant blood vessels in the eye, is vulnerable to autoimmune disease. As the mechanisms of autoimmune uveitis and corneal graft rejection are mainly related to $CD4^+$ T lymphocyte-dependent delayed hypersensitivity [11]. Then it is of worth to explore whether the iris-ciliary body plays a role in the process of corneal graft rejection.

Iris-ciliary bodys have no lymphocytes [12], but they are rich in blood vessels, and the local inflammatory reaction can induce lymphocyte migrate to the iris-ciliary body. A large number of $CD4^+$ and $CD8^+$ infiltration lymphocytes could be found in iris-ciliary body when autoimmune uveitis occurred [13]. Also, it was revealed in our preliminary studies that $CD4^+$, $CD8^+$and $CD11b^+$ T-lymphocytes infiltration occurred sequentially in iris-ciliary body and corneal graft during the process of corneal graft rejection reaction, which indicated that the sensitized lymphocytes could migrate to the corneal graft through the iris-ciliary body and the aqueous humor, and result in corneal graft rejection [14]. That might be another immune response pathway to induce corneal graft rejection. In present study, during the process of corneal graft rejection, especially the early postoperative stage, we have a more detailed tracking of $CD4^+$ lymphocytes infiltrating in iris-ciliary body, anterior chamber and corneal graft. In days 5 after surgery, $CD4^+$ lymph cells were first detected in the limbus and pars plana, then the cell lake formed in the anterior chamber angle and migrated gradually to cornea, iris and anterior chamber. We can see dense cells infiltration in the anterior chamber, iris-ciliary body and cornea grafts when immune rejection occurred. Larkin also reported that $CD4^+$ and $CD8^+$ lymphocyte infiltration could be detected in both corneal graft and anterior chamber in mice allograft rejection, and vascular endothelial cell adhesion molecule (ICAM-1) expressed in iris and corneal endothelial cells [7]. All the studies above showed that the immune cells in the corneal graft, iris-ciliary body had undergone significant changes, which further supports our speculation on the iris-ciliary body- the anterior chamber immune pathway.

Although immunological studies suggest that the iris-ciliary body is involved in the process of corneal allograft rejection, so far the study on the morphology of iris-ciliary body is scarce, especially the exploration for the ultrastructure has not been reported before. In present study, transmission electron microscopy examination revealed apparent iris edemas, increased activation and proliferation of vascular endothelial cells in iris at day 3 after allogenic corneal transplataion. And inflammatory cells in the blood vessels gradually increased followed with allograft rejection. It suggested that a significant inflammatory response occurred in iris prior to graft rejection after allogenic corneal transplantation. Large numbers of lymphocytes were found attaching to the vascular endothelium at day 7 after operation, especially lymphocytes were caught emigrating through the endothelial cell gaps, which further confirmed our prediction that the iris blood vessels might be a pathway for sensitized lymphocytes entering the anterior chamber and the corneal endothelial cells, and eventually arising endothelial allograft rejection. Following the occurrence of corneal edema, lymphocytes and monocytes in anterior chamber increased and attached to the corneal endothelium, and the corneal endothelium started to thin and aggravated gradually. This is similar with the changes of corneal endothelial cell ultrastructure in chronic dysfunction [15]. Studies had confirmed that the number of T lymphocytes, macrophages and mononuclear cells infiltrating in the aqueous humor was positively correlated with the degree of endothelial corneal graft rejection [2]. Khodadoust also found that lymphocytes activated by specific antigen could attack and destroy corneal endothelial cells, once it was injected into the anterior chamber [16]. Hence, it is not difficult to speculate that sensitized lymphocytes could reach and attack the corneal endothelial cells through the ciliary body, iris and aqueous humor, and eventually lead to endothelial immune rejection.

It had been considered that antigen presenting cells (APCs) were scarece in iris-ciliary body, and which was considered as an important cause of anterior chamber-associated immune deviation. But APCs, including plasma cells,

macrophages and mast cells had been found with meshy distribution in the iris of several kinds of animals. They are just dormant under normal circumstances [12,17,18]. Maier P. analyzed 18 samples of aqueous humor from 18 patients undergoing PK, and demonstrates that cytokine levels in the aqueous humor can be predictive for corneal graft rejection [19]. In present study, RT-PCR examination showed zero expression of CD80 and CD86 in iris-ciliary body of normal mouse and syngeneic group, while they were detected in both iris-ciliary body of allografts group at all the time points, which further confirmed the results above.

The findings of present study suggest that iris-ciliary body underwent significant inflammation after PK, and the sensitized lymphocytes could emigrate from blood vessels of iris to corneal endothelium through aqueous humor. That may be involved in endothelial corneal immune rejection. ACAID might be a special immune phenomenon under certain conditions, and it could not be induced in the cases of corneal neovascularization, infection, trauma, and suture [20]. Therefore, the role of the anterior chamber in corneal allograft rejection should not be limited to ACAID, and new research areas should be opened up especially in cases with high-risk of corneal graft rejection.

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