# AUTOPHAGY EXPLORES THE MECHANISM OF IL-1B INVOLVED IN CORNEAL ALLOGRAFT REJECTION BY REGULATING NLRP3 INFLAMMATORY BODIES

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***ABSTRACT***

***Objective:*** *To investigate the role of autophagy in the immune rejection of corneal transplantation by regulating the nucleotide binding oligomerization domain-like NLR family pyrin domain containing 3 (NLRP3) inflammatory bodies mediated by interleukin-1β (IL-1β) involved in corneal allograft rejection.*

***Methods:*** *Thirty clean and healthy male BALB/c mice and C57BL/6 mice were selected. The corneal transplantation mice were randomly divided into three groups: an allogeneic transplantation group, a rapamycin (rapa) eye drop group and a 3-methyladenine (3-mA) eye drop group. BALB/c mice were the recipients in the allogeneic transplantation group, Rapa eye drop group and 3-mA eye drop group, and C57BL 6 mice were the donors. The corneal transplantation model was established in an NLRP3 gene deficient group and wild-type mice. The recipient of the NLRP3 gene deficient group was NLRP3 gene deficient mice, and the donor was C57BL/6 mice. The donor corneal grafts were transplanted on the recipient bed. The eyes were smeared with ofloxacin eye cream after the keratoplasty. The Rapa eye drop group and 3-mA eye drop group began to take the eyes after removing the eyelid suture one day after the operation, three times a day. Fifteen rats in each group were selected the next day to observe the oedema and turbidity of corneal grafts and the growth of neovascularization in each group (allogeneic transplantation group, Rapa eye drop group, 3-mA eye drop group, NLRP3 gene defect group, wild type group). The HE staining method was used to observe changes in corneal pathology in mice in each group one month after operation. The expression of p62, LC3-I and LC3-II in mice of the allogeneic transplantation group, Rapa eye drop group and 3-mA eye drop group were observed with Western blotting. The levels of IL-1 β in serum from the allogeneic transplantation group, Rapa eye drop group and 3-mA eye drop group were measured by enzyme-linked immunosorbent assay, and the expression of IL-1 β in the lysate of mice in each group (allogeneic transplantation group, Rapa eye drop group, 3-mA eye drop group, NLRP3 gene defect group, wild type group) was measured with Western blotting.*

***Results:*** *Compared with the allogeneic transplantation group, there was no immune transplantation reaction in the Rapa group: the corneal graft was transparent, without oedema and turbidity, and there was no obvious neovascularization in the corneal graft area. In the 3-mA eye drop group, there was an immune transplantation reaction, with corneal graft oedema and thickening, turbidity, and obvious neovascularization in the corneal graft area. Compared with the allogeneic transplantation group, there was no obvious oedema in the Rapa eye drop group and less inflammatory cell infiltration in the corneal stroma; the 3-mA eye drop group had obvious oedema and a large number of inflammatory cell infiltrations in the corneal stroma. Compared with the wild-type group, NLRP3 gene deficient mice had no obvious oedema and fewer inflammatory cell infiltrations in the corneal stroma. Compared with the allogeneic transplantation group, the expression level of p62 in the Rapa eye drop group was significantly lower, and the expression level of LC3-II was significantly higher than the LC3-I. The expression level of p62 in the 3-mA eye drop group was significantly higher, and the expression level of LC3-II was significantly lower than that of LC3-I. Compared with the allogeneic transplantation group, the level of IL-1 β in the serum and lysate of the mice in the Rapa eye drop group was significantly lower, while the level of IL-1 β in the 3-mA eye drop group was significantly higher. The expression level of IL-1 β in the lysate of NLRP3 gene deficient mice was significantly lower than that of wild type mice.*

***Conclusion:*** *NLRP3 inflammatory bodies participate in corneal transplantation rejection and inhibit the transformation of IL-1 β from precursor to mature. Autophagy can mediate the involvement of IL-1 β in the inhibition of corneal allograft rejection through the regulation of NLRP3 inflammatory bodies.*

***Keywords:*** *Autophagy, NLRP3 inflammatory body, IL-1 β, corneal transplantation, immune rejection, mechanism.*

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## Introduction

Keratopathy can make the transparent cornea appear grey and white (turbid), inducing blurred vision, which is an important cause of vision loss. Previous data has indicated there are about 3.5 million patients with keratopathy in China, and the treatment rate ranks second in China's blind epidemiological adjustment, second only to cataract[(1)]. Both scar repair of a corneal injury and non-regeneration

of corneal endothelial cells have a serious impact on the treatment and prognosis of keratopathy patients. At present, however, there is no obvious and effective drug treatment. Corneal transplantation is the only effective way to treat corneal blindness, yet the main reason for corneal transplantation failure is immune rejection after corneal transplantation. The main pathological mechanism of this immune reaction is not clear[2]. The main method of prevention and treatment of corneal transplant rejection is to use an immunosuppressant, but long-term use can have harmful side effects. The establishment of stable, specific immune tolerance after organ transplantation is important to inhibit immune rejection[3]. Autophagy is a process in which the autophagy lysosome is formed by engulfing cytoplasmic proteins or organelles, enveloping them into vesicles and fusing them with lysosomes to degrade their contents. This process is present in both physiological and pathological processes of the body[4]. Autophagy has been found to maintain the central tolerance of T lymphocytes, regulate the proliferation and survival of immune cells, and plays an important role in maintaining the stability of the immune system[5]. In addition, it has been reported that autophagy can regulate the secretion of interleukin-1β (IL-1 β)[6]. Zhou et al[7] reported that autophagy can have both protective and damaging effects on renal transplantation. However, the mechanism of autophagy in corneal allograft rejection has not been well characterized. In this study, we investigated the mechanism of autophagy involving interleukin-1 β (IL-1 β) in corneal allograft rejection by regulating the NOD-like receptor family nucleotide binding oligomerization domain-like receptor 3 (NLRP3).

## Materials and methods

### *Experimental animals*

Thirty clean male BALB/c mice, C57BL/6 mice, wild type mice and NLRP3 gene deficient mice were randomly selected. All mice were purchased from Wuhan Hualianke Biotechnology Co., Ltd. (production license scxk [Shanghai] 2016-0028). Animals were housed at a temperature of 22±2°C and humidity 55±15%. Food and water was available ad libitum.

### *Main instruments and reagents*

High speed centrifuge (Shanghai Jipu Electronic Technology Co., Ltd., model: tg18kr); slit lamp microscope (Shanghai Precision Instrument Co., Ltd., model: yz5j); optical microscope (Shanghai Batuo Instrument Co., Ltd., model: XSP-1C); Venus scissors (Sihong Jinjing Medical Instrument Co., Ltd., model: 8.5cm); micro-corneal scissors (Shanghai Aitian Electronic Technology Co., Ltd.), model: 105mm; paraffin section machine (Jinhua HuaSu Technology Co., Ltd., model: hs-3090a); p62 antibody (Shanghai Kemin Biotechnology Co., Ltd.); LC3 / lc3b antibody (Beijing Taizereida Technology Co., Ltd.); IL-1 β antibody (Shanghai Yubo Biotechnology Co., Ltd.); 0.9% sodium chloride injection (Chengdu Qingshanlikang Pharmaceutical Co., Ltd., specification: 100ml: 0.9g, production batch No.: 2011120917); ofloxacin eye cream (Shenyang Qiyan Pharmaceutical Co., Ltd., production batch No.: 10940177, specification: 3.5G: 10.5mg); rapamycin nanoparticle eye drops, 3-methyladenine eye drops (all provided by the drug pharmacology laboratory of Shandong Institute of Ophthalmology); ketamine hydrochloride injection (Zhejiang Jiuxu Pharmaceutical Co., Ltd., approval No.: Gyzz h20173609, specification: 10ml: 0.1g).

### *Grouping, experimental method and observation index*

The corneal transplantation mice were randomly divided into three groups: an allogeneic transplantation group, a rapamycin (rapa) eye drop group and a 3-methyladenine (3-mA) eye drop group. BALB/c mice were the recipients in the allogeneic transplantation group, Rapa eye group and 3-mA eye group, and C57BL/6 mice were the donors. The corneal transplantation model was established in the NLRP3 gene deficient group and wild-type mice. The recipient of NLRP3 gene deficient group was NLRP3 gene deficient mice, and the donor was C57BL/6 mice.

2mg/kg ketamine hydrochloride was injected intraperitoneally for anaesthesia. After successful anaesthetic depth was attained, the cornea was fully exposed. A 2 mm diameter circular drill was used to cut the centre of the cornea. A 45° knife was used to make a side incision on one side of the cornea, and a Venus was used to cut off the cornea. The donor corneal grafts were transplanted onto the recipient bed and sutured with nylon thread. The eyes were smeared with ofloxacin, and the mice in the Rapa eye drop group and the 3-mA eye drop group began to receive eye drops after the eyelid sutures were removed one day, three times a day. The corneal suture was removed one week later.

Fifteen rats in each group were selected the next day to observe the oedema and turbidity of corneal grafts and the growth of neovascularization in each

group (allogeneic transplantation group, Rapa group, 3-mA group, NLRP3 gene defect group, wild type group). H&E staining was used to observe changes in corneal pathology of mice in each group (allogeneic transplantation group, Rapa group, 3-mA group, NLRP3 gene defect group, wild type group) one month after operation. The expression of p62, LC3-I and LC3-II in mice of the allogeneic transplantation group, Rapa group and 3-mA group were observed with Western blot. The levels of IL-1 β in the serum of the allogeneic transplantation group, Rapa eye group and 3-mA eye group were measured with enzyme-linked immunosorbent assay, and the expression of IL-1 β in the cleavage fluid of mice in each group (allogeneic transplantation group, Rapa eye group, 3-mA eye group, NLRP3 gene defect group, wild type group) was measured by Western blotting.

***Statistical methods***

SPSS18.0 software package was used for statistical data analysis, and single factor analysis of variance and an LSD-t test were used for data comparison. The degree of oedema and turbidity and the growth of neovascularization were observed with a slit lamp microscope. H&E staining was used to observe the changes in corneal pathology in mice one month after operation. The expression of autophagy related proteins p62, lc3-i, LC3-II and IL-1 β in lysate were observed by Western blot. The level of IL-1 β in serum of mice was determined by enzyme-linked immunosorbent assay. The statistical results showed that $P<0.05$ was statistically significant.

## Results

***The degree of oedema and turbidity of corneal grafts and the growth of neovascularization in each group of mice***

Compared with the allogeneic transplantation group, there was no immune transplantation reaction in the Rapa eye drop group: the corneal graft was transparent, without oedema and turbidity, and there was no obvious neovascularization in the corneal graft area. In the 3-mA eye drop group, there was an immune transplantation reaction: corneal graft oedema and thickening were present as well as turbidity, and there was obvious neovascularization in the corneal graft area. Compared with the wild-type group, the corneal grafts of the NLRP3 gene deficient group were transparent without oedema and turbidity, and there was no obvious neovascularization in the corneal grafts. See Figure 1.

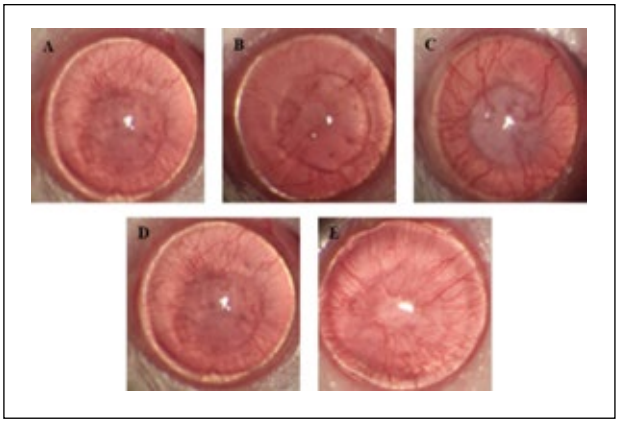


**Figure 1:** The degree of oedema and turbidity of corneal grafts and the growth of neovascularization in each group of mice.

*A: allogeneic transplantation group; B: Rapa eye drop group; C: 3-mA eye drop group; D: wild type group; E: NLRP3 gene deficient group.*

***Pathological corneal changes of mice in each group***

Compared with the allogeneic transplantation group, there was no obvious oedema in the Rapa eye drop group and less inflammatory cell infiltration in the corneal stroma.

In contrast, the 3-mA eye drop group had obvious oedema and a large number of inflammatory cell infiltrations in the corneal stroma. Compared with the wild-type group, NLRP3 gene deficient mice had no obvious oedema and less inflammatory cell infiltrations in the corneal stroma. See Figure 2.

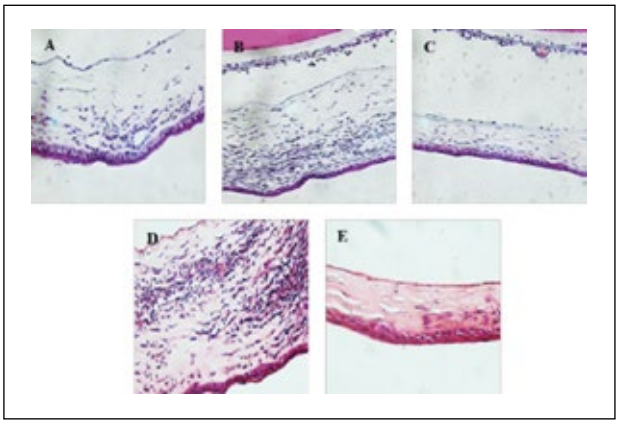


**Figure 2:** Pathological corneal changes of mice in each group.

*A: allogeneic transplantation group; B: Rapa eye drop group; C: 3-mA eye drop group; D: wild type group; E: NLRP3 gene deficient group.*

***Expression of autophagy related proteins p62, LC3-I and LC3-II in mice of each group***

Compared with the allogeneic transplantation group, the expression level of p62 in the Rapa group was significantly lower, and the expression level

of LC3-II was significantly higher than expression of LC3-I. The expression level of p62 in the 3-mA group was significantly higher, but the expression level of LC3-II was significantly lower than expression of LC3-I. See Figure 3.

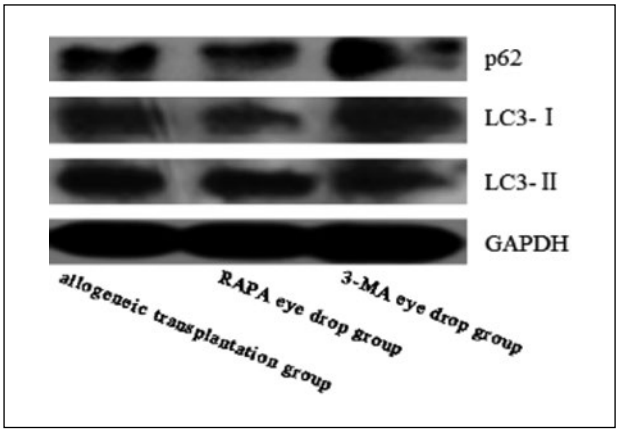


**Figure 3:** Expression of autophagy related proteins p62, lc3-i and LC3-II in mice of each group.

### *Serum IL-1 β level of mice in each group*

Compared with the allogeneic transplantation group, the level of IL-1 β in the Rapa eye drop group was significantly lower, while the level of IL-1 β in the 3-mA eye drop group was significantly higher (P<0.05). See Table 1.

| Group | n | IL-1β (pg/ml) |
|---|---|---|
| Allogeneic transplantation group | 15 | 472.52±73.66 |
| RAPA eye drop group | 15 | 83.87±44.75[a] |
| 3-MA eye drop group | 15 | 1134.49±122.91[a] |

**Table 1:** Serum IL-1 β level of mice in each group (x±s). *Note: a represents comparison with the xenotransplantation group, [a]P<0.05.*

### *Expression level of IL-1 β in macrophage lysate of mice in each group*

Compared with the xenotransplantation group, the level of IL-1 β in macrophage lysate from the Rapa group was significantly lower, while the level of IL-1 β in the 3-mA group was significantly higher. The expression level of IL-1 β in a lysate from NLRP3 gene deficient mice was significantly lower than that of wild type mice. See Figure 4.

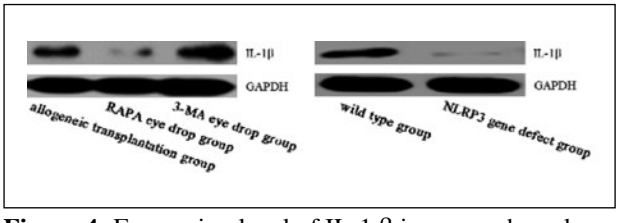


**Figure 4:** Expression level of IL-1 β in macrophage lysate from mice in each group.

## Discussion

Cornea is a relatively transparent tissue, lacking blood vessels and lymphatic vessels, which is in a relatively "forgiving state" in immunology. Keratopathy is one of the most devastating blinding eye diseases. Research has identified that there are more than 60 million patients with corneal blindness in the world, and corneal transplantation is one of the most critical methods for treating patients with corneal blindness. Corneal transplantation is a tissue transplantation procedure, which uses the normal transparent tissue of allogeneic to replace the corneal tissue with turbid lesions, with the goal of recovery or control of the corneal lesions. It is one of the most important operations in ophthalmology[8]. With the development of modern microsurgery and corneal preservation technology, rejection is still the main reason for corneal transplantation failure. Therefore, a main focus of researchers studying corneal transplantation is reducing postoperative immune rejection. Presently, the main method to inhibit immune rejection is the use of immunosuppressive drugs, but long-term use of immunosuppressive drugs will produce serious side effects, increase the risk of infection, and may even lead to cancer[9].

Rapa is a new type of macrolide immunosuppressant which can block signal transduction through different cytokine receptors and block the progression of T lymphocytes from the G1 to the S phase, so as to play the immunosuppressive effect. Rapa is a classical autophagy inducer, effectively inhibiting rapamycin target proteins and promoting autophagy[10]. The compound 3-mA is a specific autophagy inhibitor which can inhibit the activity of PI3K by interfering with the separation steps in the formation of the autophagy membrane[11].

Autophagy is a highly conserved cellular homeostasis process in eukaryote evolution. Under normal circumstances, a certain level of autophagy in cells plays an important role in maintaining the stability of the intracellular environment, which can clear pathogenic bacteria in cells and regulate the production of positive cytokines[12]. When autophagy is over-induced, it will induce programmed cell death, which is called type II programmed cell death. P62 is an autophagy-selective substrate and plays an important role in the formation of cytoplasmic protein inclusions. It has been determined that p62 levels decrease significantly during autophagy[13]. LC3 is the homolog of the yeast ATG8 gene in mammalian cells and is the only known protein related to

autophagy membrane formation. In addition, IL-1 β is an inflammatory cytokine that participates in the inflammatory process, collects immune cells and induces the production of inflammatory cytokines. It has been reported that inhibition of autophagy can promote the secretion of IL-1 β, which is an important indicator for monitoring graft rejection[14].

Inflammatory bodies are complex macromolecular protein complexes. NLRP3 inflammatory corpuscles belong to the NLR family, which are mainly composed of NLRP3, apoptosis related spot like caspase protein recruitment area and caspase-1[15]. When stimulated, it can activate cells to produce oxygen. It can activate the NLRP3 inflammatory body by releasing an active oxygen-sensitive NLRP3 ligand and its inhibitor thioredoxin, which participates in the inflammatory response[16].

The results of this study demonstrated that Rapa can inhibit the immune rejection of corneal transplantation, enhance autophagy of corneal grafts and inhibit the transformation of IL-1 β from precursor to mature, while 3-mA promoted the rejection of corneal transplantation, inhibited autophagy of corneal grafts and promoted the transformation of IL-1 β from precursor to mature. These findings therefore indicate that NLRP3 gene defects can delay corneal allograft rejection. In conclusion, NLRP3 inflammatory bodies participate in corneal transplantation rejection and inhibit the transformation of IL-1 β from precursor to mature. Autophagy can mediate the involvement of IL-1 β in inhibition of corneal allograft rejection through the regulation of NLRP3 inflammatory bodies.

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