

Kaempferol Alleviates Corneal Transplantation Rejection by Inhibiting NLRP3 Inflammasome Activation and Macrophage M1 Polarization via Promoting Autophagy

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Abstract

Corneal transplantation rejection remains a major threat to the success rate in high-risk patients. Given the many side effects presented by traditional immunosuppressants, there is an urgency to clarify the mechanism of corneal transplantation rejection and to identify new therapeutic targets. Kaempferol is a natural flavonoid that has been proven in various studies to possess anti-inflammatory, antioxidant, anticancer, and neuroprotective properties. However, the relationship between kaempferol and corneal transplantation remains largely unexplored. To address this, both *in vivo* and *in vitro*, we established a model of corneal allograft transplantation in Wistar rats and an LPS-induced inflammatory model in THP-1 derived human macrophages. In the transplantation experiments, we observed an enhancement in the NLRP3 / IL-1 β axis and in M1 macrophage polarization post-operation. In groups to which kaempferol intraperitoneal injections were administered, this response was effectively reduced. However, the effect of kaempferol was reversed after the application of autophagy inhibitors. Similarly, in the inflammatory model, we found that different concentrations of kaempferol can reduce the LPS-induced M1 polarization and NLRP3 inflammasome activation. Moreover, we confirmed that kaempferol induced autophagy and that autophagy inhibitors reversed the effect in macrophages. In conclusion, we found that kaempferol can inhibit the activation of the NLRP3 inflammasomes by inducing autophagy, thus inhibiting macrophage polarization, and ultimately alleviating corneal transplantation rejection. Thus, our study suggests that kaempferol could be used as a potential therapeutic agent in the treatment of allograft rejection.

Declarations

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Conflict of Interest (COI) statement

The authors declare no conflicts of interest.

Availability of data and material

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Code availability

Not Applicable

Authors' contributions

Huiwen Tian: Research design, Research performance, Data analysis, Article writing.

Shumei Lin: Research design; Research performance; Contributed new analytic tools.

Jing Wu: Research design; Article writing.

Ming Ma: Research performance; Article revision.

Jian Yu: Bibliography retrieval.

Yuanping Zeng: Research performance.

Qi Liu: Data analysis.

Ethics approval

All animals were kept in a pathogen-free environment and fed ad lib. The procedures for care and use of animals were approved by the Ethics Committee of the Southern Medical University and all applicable institutional and governmental regulations concerning the ethical use of animals were followed.

Consent to participate

Not Applicable.

Consent for publication

Written informed consent for publication was obtained from all participants.

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None

Introduction

Corneal blindness is the second most common form of blindness following cataracts worldwide[1]. Corneal transplantation is currently the only treatment option available[2]. However, the incidence of postoperative immune rejection is still as high as 50% on inflammatory and vascularized beds[3]. Meanwhile, immunosuppressants, the main drugs used to prevent corneal transplantation rejection, among them, corticosteroids have been shown to improve graft survival in high-risk recipients[4]. However, although immunosuppressive therapy is effective, it has many side effects, such as increasing the risk of infection, accelerating cataract formation, inducing glaucoma, which far exceed the improvement of graft survival on health[5]. Therefore, it is urgent to explore the mechanisms involved in corneal allograft rejection so as to identify novel therapeutic targets.

Macrophages are involved in the initial stages of corneal transplantation rejection[6]. It is generally suggested that they can be divided into the M1 and M2 phenotypes induced by external stimulation[7]. M1 macrophages highly express CD80 and CD86 receptors and secrete IL-6, iNOS and other pro-inflammatory cytokines, whereas M2 macrophages highly express CD206 receptors and predominantly secrete IL-10 and other anti-inflammatory cytokines[8].

The NLR family pyrin domain-containing 3 (NLRP3) inflammasome is a member of the pathogen recognition receptors (PRRs) and is widely expressed in macrophages. It is involved in the *in vivo* recognition of danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) and the subsequent triggering of inflammatory responses[9]. Activation of the NLRP3 inflammasome transforms precursor Caspase-1 into Caspase-1, which then cleaves the precursor of IL-1 β into a mature form and releases it to the downstream immune inflammatory reaction cascade[10]. The NLRP3 inflammasome has been proven to participate in immune rejection post-transplantation in graft-versus-host disease (GVHD), as well as heart, kidney, and skin transplantations[11, 12]. Studies have shown that the NLRP3 inflammasome is associated with the M1 polarization of macrophages[13].

Autophagy is a process of self-digestion in higher eukaryotes, and is associated with many physiological and pathological processes[14]. Some recent studies have shown that enhanced myocardial autophagy can delay the occurrence of heart transplantation rejection[15]. Kaempferol (Ka) is a natural flavonoid small molecule, and is the active component in various traditional Chinese medicines[16]. It has been proven to have anti-inflammatory, antioxidant, anticancer and neuroprotective effects[17]. However, the role and mechanism of kaempferol in corneal allograft rejection remain unknown. In this study, we explored the effects of kaempferol on corneal allograft rejection in rats and used an inflammatory model in human macrophages to further investigate whether these are in fact mediated by autophagy.

Material And Methods

2.1. Animals

Specific pathogen-free (SPF) animals were selected for our experiment. Specifically, 50 Sprague Dawley (SD) and 112 Wistar rats, which were all female, 6-8 weeks old; and all weighing 180–220 g. Among them, 40 Wistar rats were used for survival analysis, 30 were used for mRNA expression analysis, and 30 were used for western blot analysis. Twelve Wistar rats were used for immunofluorescence staining. The rats were procured from the Guangdong Medical Experimental Animal Center and raised in the experimental animal center of Nanfang Hospital of Southern Medical University (temperature 18–25 °C, humidity 40%, light/dark time: 12/12 h).

2.2. Ethic statement

The animal experiment plan has been reviewed and approved by Nanfang Hospital Animal Ethic Committee, which is in line with the principles of animal protection, animal welfare and ethics, and the relevant provisions of the national experimental animal ethical welfare. The feeding and use of the experimental animals were in accordance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals.

- **Establishment of the Rat Corneal Allograft Model**

The Wistar rats were divided into the following groups: control, corneal autograft, corneal allograft, kaempferol, vehicle control, and kaempferol + 3-MA (kaempferol combined with the autophagy inhibitor 3-methyladenine). The corneal allograft group was then further divided into three subgroups named Day 5, Day 9, and Day 14, according to the time of sampling post-operation.

Autogenous (Wistar→Wistar) and allogeneic keratoplasty (SD→Wistar) models were established according to the methodology of Williams et al. [18]. The rejection index (RI) was calculated according to the degree of opacification, edema, and vascularization of the corneal graft according to the scoring standards of Larkin[19] (0–4, 0–2, and 0–4, respectively). Rejection was defined as $RI \geq 5$ or an opacification degree ≥ 3 .

2.4. Establishment of the LPS-induced macrophage inflammatory model

The human monocytic leukemia cell line (THP-1) was purchased from the cell bank of the Chinese Academy of Sciences. The cells were maintained in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin (Gibco, USA), at 37 °C, in a 5% CO₂ atmosphere. The cell line was centrifuged and resuspended in culture medium containing phorbol 12-myristate 13-acetate (PMA) (100 ng/mL) (Sigma Aldrich, USA). After 48 h of induction with PMA, the adherent cells were considered M0 macrophages.

The THP-1 derived macrophages were divided into four groups according to experimental needs: (1) the control group (macrophages with no treatment); (2) the lipopolysaccharide (LPS) group (1 µg/mL LPS treatment for 24 h; Sigma Aldrich); (3) the kaempferol group (1 µg/mL LPS + kaempferol treatment for 24 h); and (4) the kaempferol + 3-MA group (1 µg/mL LPS + 40µM kaempferol + 5mM 3-MA treatment for 24 h).

2.5. Administration of kaempferol and 3-MA

In the animal experiments, kaempferol (Selleck, USA) was first prepared into a 200 mg/mL solution with DMSO and then diluted into a 10 mg/mL working solution with a vehicle (DMSO + polyethylene glycol + polyoxyethylene sorbitan mono-oleate + pure water) before injection. Rats in the kaempferol groups were intraperitoneally injected with 50 mg/kg of kaempferol working solution every day, starting at 3 days before the operation and continuing till the date of sampling or till the end of observation (which occurred either at the instance of graft rejection, or else 30 days after the operation). Rats in the vehicle control

groups were injected with 5 mg/kg of the aforementioned vehicle daily. Rats in the kaempferol+ 3-MA group were intraperitoneally injected with 10 mg/kg of 3-MA solution (Selleck) 12 h before the kaempferol injection.

In the cell experiments, kaempferol was diluted to 40 mM with DMSO and incubated with macrophages at a dilution rate of 20 to 200 μ M, while 3-MA was diluted to 50 mM with ultrapure water and incubated with macrophages at 5 mM.

- **Cell Counting Kit-8 (CCK-8) Assay**

Macrophages were incubated with different concentrations of kaempferol (0, 20, 40, 100, and 200 μ M) for 12 and 24 h. After that, the instructions of the cell-counting kit-8 (CCK-8 Kit; Dojindo, Japan) were followed for the assay. Absorbance was measured at 450 nm by fluorometer, and the cell survival rate was calculated according to the instrument manufacturer .

- **Reverse Transcriptase Real-Time Polymerase Chain Reaction (RT-PCR)**

In accordance with the operating instructions, RNAiso Plus (Trizol) (TaKaRa, Japan) was used to extract total RNA from the rat corneas (one piece of cornea per group) and the macrophages. PrimeScript RT Master Mix (TaKaRa) was used to reverse transcribe the mRNA into cDNA. The PCR system was prepared according to the 2 \times SYBR Green qPCR Master Mix (Selleck) instructions. The LightCycler 480 system was performed to detect the mRNA content of each sample. Melting curves were analyzed to confirm the homogeneity of the product. The primers used were synthesized by Sangon Biotech and are shown in Table 1. Rat and human GAPDH (Sangon Biotech, China) are provided for internal reference.

- **Western Blot Analysis**

Protein samples were obtained from the rat corneas (one piece of cornea per group) and the THP-1 macrophages, and were lysed using RIPA lysis buffer (Beyotime, China). The protein concentrations were then determined using a BCA protein assay kit (Beyotime), wherein 20-30 μ g of protein was loaded each time and separated by 10-15% SDS-PAGE, then transferred to poly(vinylidene fluoride) (PVDF) membranes. The membranes were then blocked with 5% non-fat milk for 1 h at room temperature and subsequently incubated with primary antibodies overnight at 4 $^{\circ}$ C. The dilution ratios of primary antibodies were as follows: the dilution ratio for anti-NLRP3, anti-IL-1 β , anti-caspase-1, anti-microtubule-associated protein light chain 3 (LC3), and anti-p62 (Abclonal, China) was 1:800; the dilution ratio for anti- β -actin (Fude antibody, China) was 1:10,000. Following incubation with the AffiniPure Goat Anti-Rabbit IgG (H+L) and AffiniPure Goat Anti-Mouse IgG (H+L) (Fude antibody), the films were exposed and scanned using ECL reagent (Affinity Biosciences, USA). ImageJ software (Rawak Software, Germany) was used to analyze the band gray value. The representative blots are shown in the figures.

- **Fluorescence Microscopy**

The corneal tissue sections were permeabilized with 0.5% Triton X-100, then blocked with goat serum (Solarbio, China) for 60 min. anti-NLRP3 and anti-CD80 (Santa Cruz Biotechnology, USA) were prepared at a dilution ratio of 1:100 and incubated with the cornea sections at 4 °C overnight. After incubating with the secondary antibodies at room temperature for 1 h, an anti-fade solution (containing DAPI) (Solarbio) was added, and the sections were observed under a fluorescence microscope.

Cell immunofluorescence staining was carried out by counting and inoculating the THP-1 cells in a confocal culture dish. Paraformaldehyde (4%) was added, and the cells were fixed for 15 minutes. The remaining steps were the same as described above for immunofluorescence staining of the cornea.

To observe the induction of autophagy, autophagosome-lysosome living cell dye DALGreen (Dojindo) was added to the macrophages cultured in the confocal dish for 30 min according to the manuscript. The remaining steps were the same as above for the cornea and cell immunofluorescence methods. Relative fluorescence intensity was analyzed by ImageJ software.

2.10. Flow Cytometry

Macrophages were digested with trypsin-EDTA (Gibco) in a 37 °C incubator for 5 min and then centrifuged and resuspended with PBS. Fluorescent antibodies directed against CD molecules FITC-CD11b, PE-CD80, and APC-CD206 (eBioscience, USA) were added, and the macrophages were evaluated using flow cytometry. The gating strategy incorporated live cells and singlet gates prior to gating on CD11b, after which CD80 and CD206 were detected. The results were analyzed using Flowjo (BD Falcon, USA).

2.11. Statistical Analysis

The Kaplan-Meier statistical method was used to analyze graft survival times, the time required till corneal graft rejection occurred or the observation period reached day 30 post-operation.

All data were expressed as the mean \pm SD and one-way analysis of variance (ANOVA) was used for statistical analysis. SPSS 20.0 (IBM, USA) was used for statistical analysis. Statistical significance was set at $p < 0.05$.

Results

3.1. Kaempferol inhibited NLRP3 inflammasome expression in corneal allografts and prolonged graft survival time

Starting at 7 days post-operation, neovascularization commenced around the graft in the autograft group. However, the graft remained transparent throughout the observation window (i.e., 30 days post-operation). In the allograft group, corneal edema and neovascularization began on day 7, after which the graft began to appear turbid and edematous. On day 14, acute corneal edema and a large amount of neovascularization invaded the graft, exceeding the radius by 50%. The graft was turbid, obviously

edematous, and the pupil contour was difficult to distinguish; RI reached 5, which indicated rejection (Figure 1A).

In order to investigate whether the NLR family pyrin domain-containing 3 (NLRP3) inflammasome is involved in the rejection of corneal allografts, we studied its expression in the cornea. The expression of NLRP3 and IL-1 β mRNA in the allograft group was found to be significantly higher than that in the control and autograft groups on days 5, 9, and 14 post-operation (Figure 1B). The peak expression of mRNAs was observed on day 5 post-operation.

However, in the kaempferol group, corneal edema subsided on the fifth day after operation. The cornea was still transparent on days 5, 9, and 14 post-operation, with no new blood vessel growth in the graft. Some of the grafts began to be rejected from day 26, while half remained with a RI<3 during the 30 day observation period (Figure 1C). The mRNA expression of NLRP3 and IL-1 β at day 5 was significantly lower in the kaempferol group than in the vehicle control group (Figure 1D). The same applied for the protein expression of NLRP3 and IL-1 β (Figure 1E).ndicate that kaempferol inhibited the expression of the NLRP3 inflammasome in the rat corneal allograft model.

- **Activation of autophagy is critical for kaempferol's inhibition of the NLRP3 inflammasome and alleviation of graft-rejection**

To investigate whether autophagy is involved in the therapeutic effect of kaempferol on corneal allograft rejection, the autophagy inhibitor 3-methyladenine (3-MA) was used.

In the group of rats to which kaempferol only was administered, expression of the autophagy genes Beclin1, LC3, and ATG5 was upregulated and that of p62 mRNA was downregulated compared with that in the control and vehicle control groups, indicating the activation of autophagy. Following treatment with a combination of kaempferol and 3-MA, the expression of these mRNAs was significantly reversed, indicating that autophagy was inhibited (Figure 2A). Parallely, all the grafts was rejected at day 14 in the kaempferol+3-MA group (Figure 2B). Compared with the allograft (12.6 ± 1.08 days), vehicle control (11.4 ± 0.97 days), and kaempferol + 3-MA (10.5 ± 0.71) groups, the corneal graft survival time of the kaempferol group was significantly prolonged to (27.7 ± 1.49) days (Figure 2C).

After the combined application of kaempferol and 3-MA, the mRNA and protein expression of NLRP3 and IL-1 β was significantly higher than that in the kaempferol group on day 5 post-operation (Figure 2D, E), indicating that the inhibitory effect of kaempferol on the NLRP3 inflammasome was weakened by the addition of the autophagy inhibitor. Combined with the above results, this suggests that autophagy is critical for kaempferol's inhibition of the NLRP3 inflammasome and alleviation of transplant rejection.

- **Kaempferol inhibits M1 polarization in cornea by inhibiting the activation of the NLRP3 inflammasome**

Firstly, to verify the role of macrophage polarization in the occurrence of rejection after corneal transplantation, we used RT-PCR to detect the expression of cornea M1 cytokines in the isograft and

allograft groups. The mRNA expression levels of M1 cytokines iNOS, IL-6, TNF- α , CXCL-10 in the allograft group were significantly upregulated on days 5, 9, and 14 post-operation compared with the isograft group (Figure 3A).

Secondly, we explored the effect of kaempferol on macrophage polarization after corneal transplantation. On day 5 post-operation, the mRNA expression levels of the M1 cytokines in the kaempferol group were significantly lower than those in the allograft group. This indicates that kaempferol treatment can effectively inhibit cornea M1 polarization. However, after the combined use of 3-MA, the mRNA expression levels of the M1 cytokines were significantly up-regulated, which suggests that the effect of kaempferol on macrophage polarization is related to autophagy (Figure 3B).

To further explore the relationship between the expression of the NLRP3 inflammasome and M1 polarization, we studied the expression of NLRP3 and CD86 in each group using immunofluorescence. Compared with the control group, the fluorescence intensity of CD86 and NLRP3 of the allograft group was significantly increased, with co-staining shown correlation between NLRP3 and CD86. The relative fluorescence intensity of CD86 and NLRP3 in the cornea in the kaempferol group was decreased (Figure 3C). These results suggest that M1 polarization and NLRP3 inflammasome expression both increase after allograft keratoplasty and that kaempferol can effectively reduce this response.

- **Kaempferol decreases LPS-induced M1 polarization *in vitro***

In order to further study the mechanism of kaempferol inhibition of M1 macrophage polarization, we directly carried out cell experiments on macrophages. The results of the cell-counting kit-8 (CCK-8) assay revealed that kaempferol acted in a dose-dependent manner in both the 12 h and 24 h groups. However, the number of surviving cells was still more than 50% after 24 h administration with 200 μ M of kaempferol (Figure 4A).

Three concentrations of kaempferol had been tested: 20 μ M, 40 μ M, and 100 μ M. After LPS treatment, the relative mRNA expression of M1-associated cytokines IL-6, iNOS, TNF- α and CXCL-10 was upregulated. According with the result *in vivo*, kaempferol at different concentrations significantly downregulated the expression of all mRNAs (Figure 4B).

Next, we observed the effect of kaempferol on the proportion of M1 and M2 macrophages using flow cytometry. The expression of CD11b, CD80, and CD206 was used to detect the distinct macrophage phenotypes. Among them, CD11b⁺ CD80⁺ cells were considered to be M1 macrophages, whereas CD11b⁺ CD206⁺ cells were considered to be M2 macrophages. The results showed that the ratio of M1 to M2 in the LPS group significantly increased compared with the control group. Following kaempferol treatment, however, the above effect was significantly reversed, and the M1: M2 ratio was decreased (Figure 4C). These results suggest that kaempferol can reduce the polarization of M1 macrophages induced by LPS.

- **Kaempferol reduces the activation of the NLRP3 inflammasome in macrophages *in vitro***

The RT-PCR results showed that the mRNA expression of NLRP3, Caspase-1, and ASC was upregulated after LPS treatment in macrophages. Subsequent kaempferol treatment at different concentrations significantly downregulated it (Figure 5A). Additionally, kaempferol at all concentrations significantly decreased the protein levels of NLRP3, pro-IL-1 β , IL-1 β , pro-Caspase-1, and Caspase-1 (which had been increased by LPS) (Figure 5B). Furthermore, NLRP3 protein expression and CD86 positive cells decreased significantly after kaempferol treatment, which was consistent with the experimental results *in vivo* (Figure 5C).

- **Kaempferol promotes autophagy *in vitro***

Autophagy inhibitor 3-MA was used to reduce autophagy in macrophages. The results of RT-PCR showed that kaempferol at different concentrations significantly upregulated the mRNA expression of LC3, and downregulated that of p62 (Figure 6A). Accordingly, western blotting revealed the same pattern, and the LC3 II/I ratio was increased compared with that of the LPS group. The combination of the autophagy inhibitor 3-MA and kaempferol resulted in a significant decrease in the LC3II/I ratio and an increase in p62 expression, thus reversing the effects of kaempferol alone (Figure 6B).

DALGreen and LC3B co-staining was also produced. The results of the co-staining showed that the relative fluorescence intensity of DALGreen and LC3 were significantly increased after kaempferol administration. Conversely, DALGreen and LC3 fluorescence intensity was weakened after 3-MA was added (Figure 6C).

In line with what our *in vivo* findings indicated, the results of the above tests confirm that kaempferol can indeed enhance the autophagy of macrophages.

3.7. Autophagy inhibitors can inhibit the effects of kaempferol on macrophage polarization and NLRP3 inflammasome activation *in vitro*

Finally, we considered the effect of autophagy inhibitors on kaempferol's inhibition of the NLRP3 inflammasome and of macrophage polarization.

Following treatment with 3-MA, the expression of NLRP3, pro-Caspase-1, Caspase-1, pro-IL-1 β and IL-1 β protein increased relative to that in the kaempferol-only group, indicating that 3-MA inhibits the inhibitory effect of kaempferol on the NLRP3 inflammasome (Figure 7A).

In terms of what concerns macrophage polarization, the results of flow cytometry showed that, compared with the kaempferol group, the M1/M2 ratio in the kaempferol + 3-MA group increased significantly, indicating that autophagy participates in the process of macrophage polarization (Figure 7B). In further support of this, results of NLRP3/CD86 co-staining showed that the fluorescence intensity of NLRP3 and the proportion of CD86 positive cells in kaempferol groups increased after 3-MA treatment (Figure 7C). Together, these results demonstrate that the effects of kaempferol on macrophage polarization and the NLRP3 inflammasome was via inducing autophagy.

Conclusions

Macrophages are the main components of the innate immune system and constitute the first barrier of immune defense[20]. Studies have shown them to be pluripotent cells with high plasticity and functional diversity that can polarize towards different phenotypes[21]. Before the phenomenon of macrophage polarization was found, the role of APC headed by macrophages in corneal transplantation immunity was collectively referred to as the costimulatory pathway. This mainly includes the interaction between CD28 on T cells and B7 molecules (CD80, CD86) on APCs[22]. It is believed that activated macrophages are inflammatory cells with an antigen presentation function and a costimulatory function[23]. However, after the emergence of the concept of macrophage polarization, studies suggest that M1 macrophage cell membranes highly express HLA-DR, CD80, CD86, and other protein markers, and participate in pro-inflammatory reactions[24]. Therefore, we have reason to think that M1 macrophage polarization is involved in corneal transplantation rejection, and that M1 macrophages are the main force responsible for secreting chemokines and presenting antigens. Our experiment corroborates these hunches, showing that M1 cytokines increased following corneal allograft transplantation.

Our study also investigated the potential therapeutic effect of kaempferol on macrophage polarization in corneal transplantation. We found that in groups to which kaempferol was administered, M1 cytokines were down regulated. This indicated that kaempferol could inhibit the aggregation of M1 macrophages after corneal transplantation. Additionally, using an inflammation model in macrophages, we showed that kaempferol at different concentrations had the same inhibitory effect on M1 polarization in human cells *in vitro*.

The NLR family, pyrin domain-containing 3 (NLRP3) inflammasome is composed of the NLRP receptor, apoptosis-related spot-like protein (ASC), and pro-caspase-1[25]. Many studies have shown that the NLRP3 inflammasome can induce macrophages into the M1 phenotype. For example, knockout of the NLRP3 gene or use of NLRP3 inhibitor CY09 can effectively inhibit M1 polarization[26]. Similarly, the NLRP3 inflammasome induces M1 polarization in oral inflammation, ischemic stroke, and other diseases[27, 28]. In our study, we confirmed that the NLRP3 inflammasome and downstream cytokine IL-1 β were aggregated in the corneal epithelium post-transplantation in the rat model, indicating once again that the NLRP3/IL-1 β axis is involved in the early stage of corneal allograft rejection.

Scholars have defined that reactive oxygen species (ROS) is the bridge between the NLRP3 inflammasome and M1 macrophage polarization[29]. In the process of mitochondrial dysfunction, released ROS-activated NLRP3 inflammasome and pro-caspase-1 (an important component of the NLRP3 inflammasome) cleave into caspase-1, which contributes to the production and secretion of M1 proinflammatory cells[30]. Meanwhile, M1 macrophage polarization can in turn promote the activation of the NLRP3 inflammasome, contributing to the production of high-level proinflammatory cells.

Inflammatory factors and ROS can increase and maintain the inflammatory response[13]. In ophthalmology research, studies have shown the increase in ROS to be involved in the occurrence of corneal allograft rejection. The inhibition of mitochondrial damage, however, prolonged the survival of

corneal grafts[31]. Therefore, we speculated that mitochondrial damage may be the cause of NLRP3 inflammasome activation after corneal transplantation. In our animal and cell experiments, we demonstrated that kaempferol at different concentrations can reduce the activation of the NLRP3 inflammasome and the expression of IL-1 β and caspase-1.

Autophagy promotes the degradation of misfolded proteins, abnormal organelles, and other waste materials while maintaining cell metabolic function[32]. Microtubule-associated protein light chain 3 LC3 and p62 are critical proteins in autophagy. In particular, cytoplasmic soluble LC3 (LC3I) is hydrolyzed during the autophagic process to form the membrane-bound LC3 (LC3II), with the increase in LC3II/I thus being indicative of enhancement of autophagy[33, 34]. P62 binds to ubiquitinated proteins and interacts with LC3, mediating ubiquitinated protein transport in the autophagic progress[35]. Autophagy plays an important role in the regulation of the inflammatory response [36, 37]. The decrease in autophagy leads to a large number of depolarized mitochondria and leaked substances, including mitochondrial DNA and ROS[38, 39]. In addition, there is increasing evidence to suggest that moderating autophagy can promote the polarization of macrophages from the M1 to the M2 phenotype, while the ensuing deficiency of autophagy-related genes could drive the upregulation of M1 polarization[40-42].

We found that autophagy is key to kaempferol inhibiting the NLRP3 inflammasome and M1 polarization, both *in vivo* and *in vitro*. In our animal model of corneal transplantation, kaempferol promoted an increase in autophagy-related mRNA in corneal tissue, as well as the conversion of LC3I to LC3II in macrophages, and reduction in the accumulation of p62. To further explore the role of autophagy in kaempferol treatment, we used the autophagy inhibitor 3-methyladenine (3-MA), which selectively inhibits phosphatidylinositol-3 kinase (PI3K) and thus inhibits the initial stage of autophagy[43]. We found that 3-MA attenuated the protective effect of kaempferol on corneal allograft rejection. Similarly, *in vitro*, 3-MA blocked the effects of kaempferol, significantly increasing the proportion of M1 macrophages and activating the NLRP3 inflammasome. Finally, we observed the induction of autophagy using DALGreen, a lipid soluble fluorescent dye that enters the cell membrane and can be used to detect autophagosomes[44]. We found that the fluorescence intensity of DALGreen and LC3 was significantly enhanced by kaempferol, and the staining sites were highly consistent. As expected, 3-MA weakened the fluorescence intensity. This further confirmed that promotion of autophagy is in fact the mechanism by which kaempferol inhibits macrophage polarization and NLRP3 inflammasome activation.

In conclusion, our study demonstrated that kaempferol can inhibit the activation of the NLRP3 inflammasome and M1 polarization via activating autophagy, thus delaying the occurrence of corneal allograft rejection *in vivo* and reducing inflammation in macrophages *in vitro*. We acknowledge that there are some shortcomings in our experiments; for example, we mainly focused on the role of kaempferol on macrophages. Previous studies suggested that kaempferol promotes the proliferation of CD4 β FoxP3 β regulatory T cells, thereby affecting islet transplantation rejection[45]. In addition, we found that the rats inoculated with kaempferol had partial rejection near the end of the observation. Therefore, the role of kaempferol and other immune cells in corneal transplantation should be explored, and we consider that further study on the administration mode and dosage of kaempferol may have a deeper impact on the

graft survival rate. We believe that these qualities of chemo-preventive activity and *in vivo*-tolerance in kaempferol highlight it as a novel therapeutic option for the prevention of transplantation rejection in humans.

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Abbreviations

Ka, kaempferol; LC3, Microtubule-associated protein light chain 3; APC, Antigen presenting cell; M1 and M2, distinct macrophage phenotypes; 3-MA, 3-methyladenine; NLRP3 inflammasome, NLR family pyrin domain-containing 3 inflammasome; THP-1, human monocytic leukaemia cell line; ROS, Reactive oxygen species.

Tables

Table 1. Sequence of the primers for RT-PCR

mRNA	Forward primer (5'→3')	Reverse primer (5'→3')
Rat NLRP3	GGCTGCGGATGGAATTTGGA	ATCTCTCGCAGTCCACCTCC
Rat IL-1 β	TGGGATGATGACGACCTGCT	GCTCACATGGGTCAGACAGC
Rat IL-6	TGAAACCCTAGTTCATATCTTCAAACAA	GCCACTCCTTCTGTGACTCTAACTT
Rat iNOS	AGCTCGGGCTGAAGTGGTAT	CTCGGTGCCCATGTACCAAC
Rat TNF- α	AGTTCCCAAATGGGCTCCCT	TGGTGGTTTGCTACGACGTG
Rat CXCL-10	TGAAAGCGGTGAGCCAAAGA	CTAGCCGCACACTGGGTAAA
Rat Beclin1	GATCCTGGACCGAGTGACCA	AGACACCATCCTGGCGAGTT
Rat LC3B	CCCCACCAAGATCCCAGTGA	TTAGCATTGAGCTGCAGGCG
Rat Atg5	GTGTGAAGGAAGCTGACGCT	CCATGAGTTTCCGGTTGATGGT
Rat p62	CATCTCCCACCAGAGGCTGA	TGGTCTGTAGGAGCCTGGTG
Rat GAPDH	GAAGGTCGGTGTGAACGGAT	CCCATTTGATGTTAGCGGGAT
Human NLRP3	TGCCCAGACATCCAAGACCA	CCATCTGCAGCCAAAGAGCA
Human Caspase-1	CCGTTATTCCGAAAGGGGCA	TCTGAGCCTGAGGATGTGGG
Human ASC	ACCCAAGCAAGATGCGGAAG	TTAGGGCCTGGAGGAGCAAG
Human IL-6	ACACAGACAGCCACTCACCT	TTCTGCCAGTGCCTCTTTGC
Human iNOS	AGCCTGAGAGACAGAGGCTG	CTGGGAAAGCAGGAAGCCAG
Human TNF- α	GGCAGGTTCTGTCCCTTTCA	GTCGCGGATCATGCTTTCTG
Human CXCL-10	TGCCATTCTGATTTGCTGCC	TGCAGGTACAGCGTACAGTT
Human LC3B	CAGGCCTTCTTCCTGTTGGTG	TCATCCCGAACGTCTCCTGG
Human p62	GTCCCTCTCCAGATGCTGT	AGCCGCTCCGATGTCATAGT
Human GAPDH	GGAGTCCACTGGCGTCTTCA	GTCATGAGTCCTTCCACGATACC

Figures

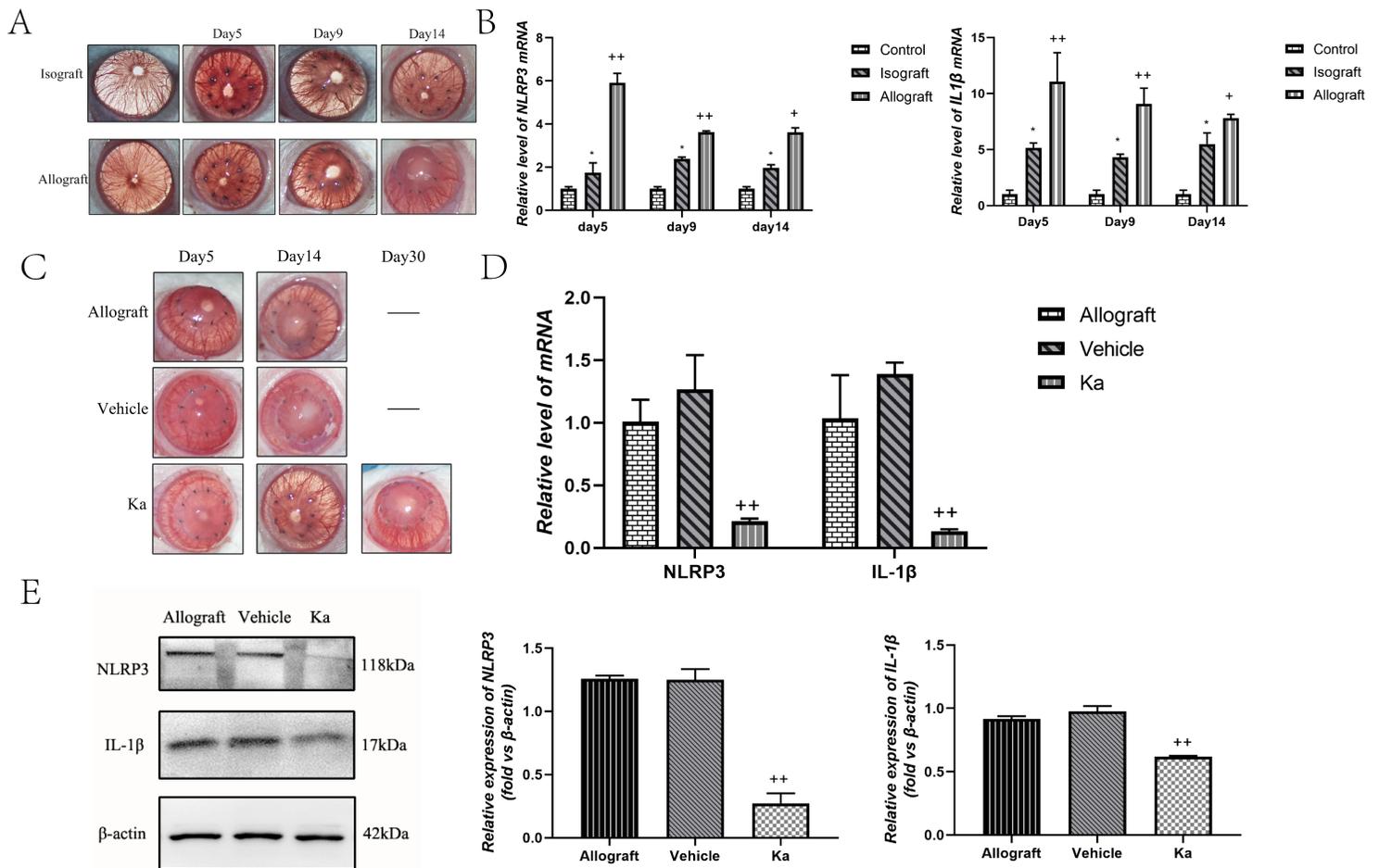


Figure 1

Kaempferol inhibited the activation of NLRP3 inflammasome post-transplantation. (A) Corneal morphology under microscope at different time points post-transplantation in isograft and allograft. (B) Rats were sacrificed at days 5, 9 and 14 post-operation and relative cornea mRNA level of NLRP3 and IL-1β between control, isograft and allograft group are detected by qRT-PCR. (C) Kaempferol working solution (50mg/kg) was injected intraperitoneally daily, the corneal grafts were observed on the days 5, 9 and 14 post-operation. (D) Rats were sacrificed at day 5 post-operation and relative cornea mRNA level of NLRP3 and IL-1β between allograft, vehicle and kaempferol group are detected. (E) Rats were sacrificed at day 5 post-operation and relative protein level of cornea NLRP3 and IL-1β between allograft, vehicle and kaempferol group were detected by western blot analysis. One-way ANOVA was used and values are shown as means ± SD, n = 3–4 for each group. *p < 0.05 vs control group; +p < 0.05, ++p < 0.01 vs isograft group (B); +p < 0.05, ++p < 0.01 vs vehicle group (D, E).

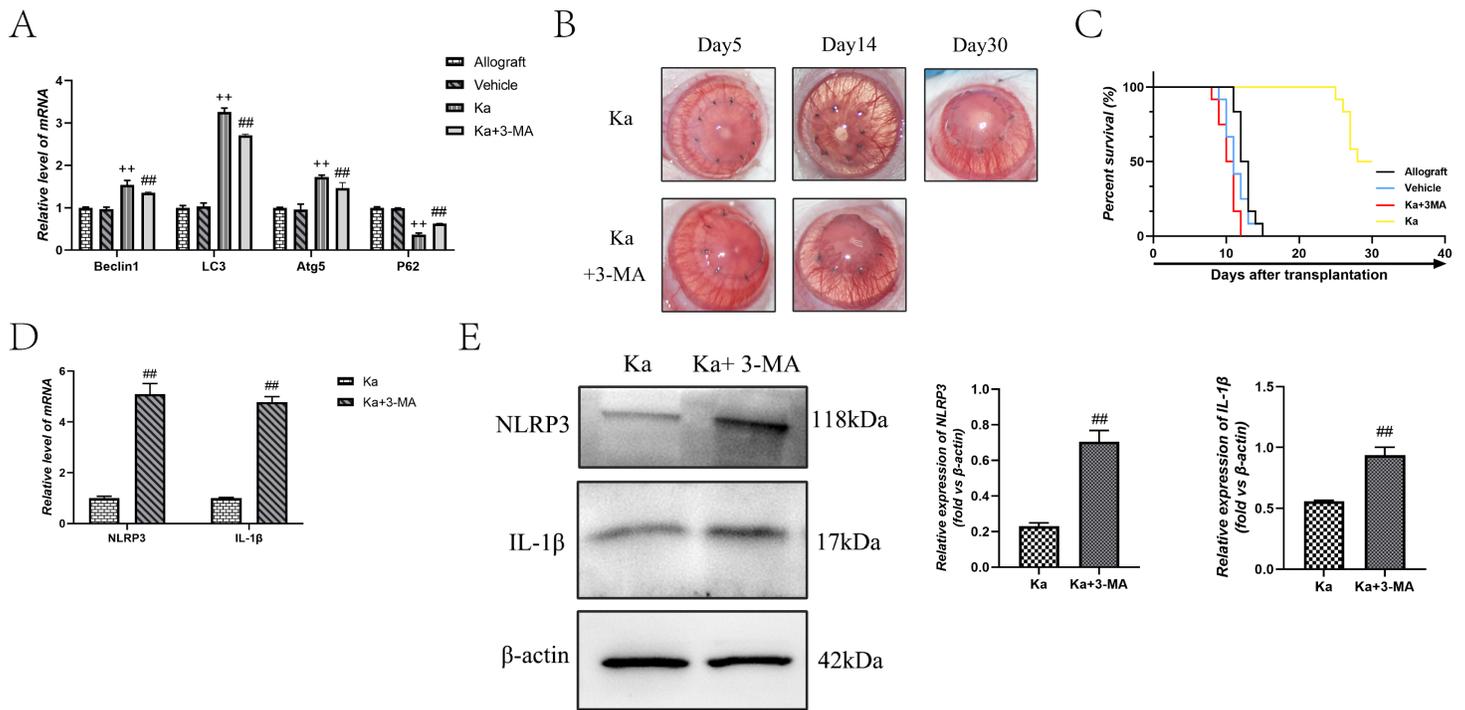


Figure 2

Autophagy relate in the anti-rejection effect of kaempferol. (A) Rats post-transplantation were treated by vehicle, kaempferol or kaempferol and 3-MA (10mg/kg), and the corneas were taken at day 5, the relative cornea mRNA level of autophagy related genes were detected. (B) Rats were treated with kaempferol or kaempferol and 3-MA, the corneal grafts were observed on the days 5, 9 and 14 post-operation. (C) The survival curves of graft between allograft, vehicle, kaempferol and kaempferol + 3-MA group (10 Wistar rats per group were used to observe the graft survival time). (D) Relative cornea mRNA level of NLRP3 and IL-1β between kaempferol and kaempferol plus 3-MA group are detected at day 5. (E) Relative protein expression of cornea NLRP3 and IL-1β in kaempferol and kaempferol + 3-MA group were detected by western blot analysis at day 5. One-way ANOVA was used and values are shown as means ± SD, n = 3–4 for each group. ++p < 0.01 vs vehicle group; ##p < 0.01 vs kaempferol group.

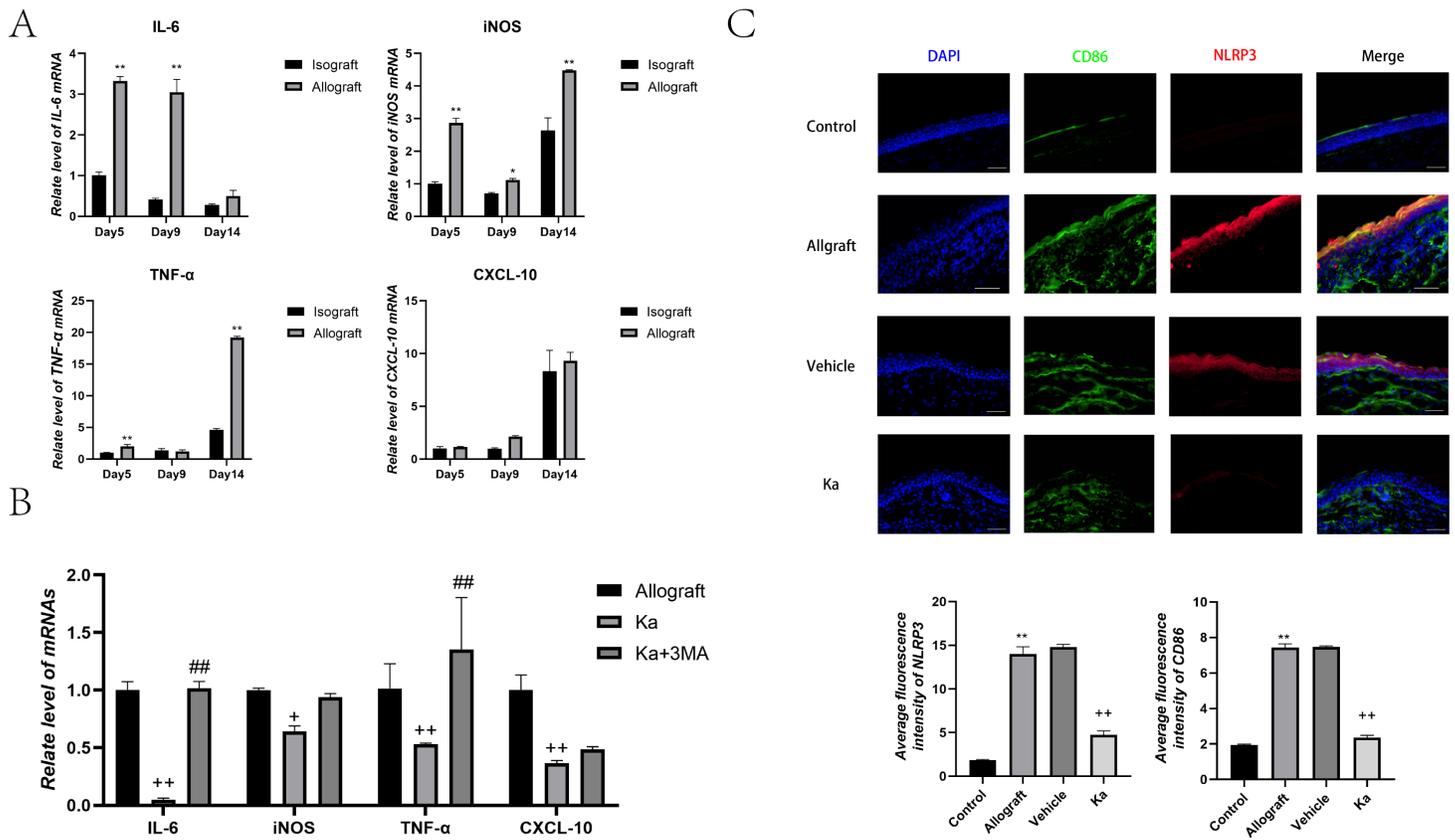


Figure 3

Kaempferol inhibited the M1 polarization post-transplantation. (A) Rats in allograft were divided into Day 5, Day 9, Day 14 sub-groups, the relative cornea mRNA level of M1 cytokines between control, Day 5, Day 9 and Day 14 group are detected. (B) The relative cornea mRNA level of M1 cytokines between allograft, kaempferol and kaempferol+3-MA group are detected at day 5. (C) The rat cornea in control, allograft, vehicle and kaempferol group was taken at day 5 and made into frozen sections, CD86 (green) and NLRP3 (red) were detected by fluorescence microscopy. DAPI (blue) was used as a nuclear dye. Scale bar, 50µm. One-way ANOVA was used and values are shown as means ± SD, n = 3–4 for each group. *p < 0.05, **p < 0.01 vs control group; +p < 0.05, ++p < 0.01 compared with allograft group; ##p < 0.01 compared with kaempferol group.

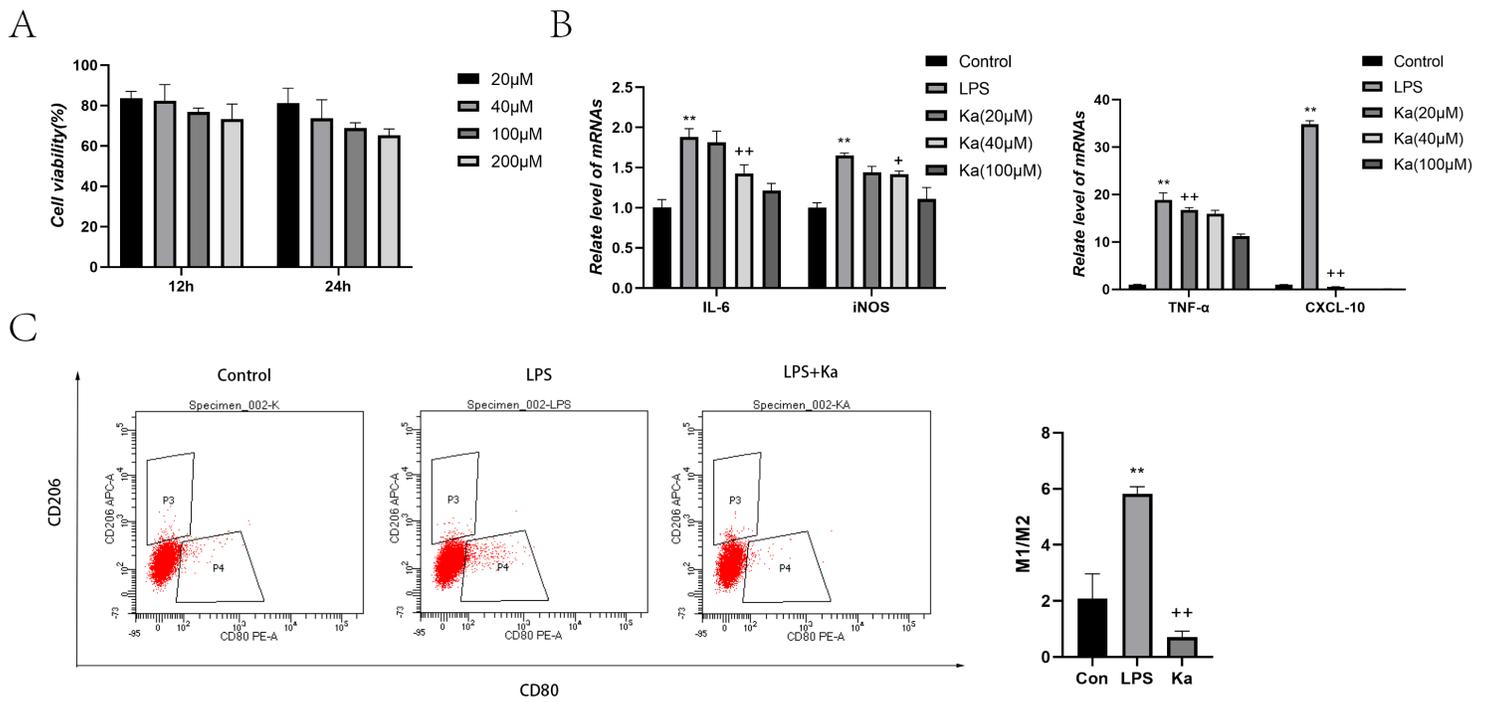


Figure 4

Kaempferol inhibited the M1 polarization in THP-1 derived macrophages. (A) The toxic effects of kaempferol concentration (from 20 μM to 200 μM) and action time (12h, 24h) on macrophages were detected by CCK-8 assay. (B) LPS (1 μg/mL) is used to create an inflammatory environment to promote macrophage M1 polarization. Kaempferol in different concentration (20, 40, 100 μM) were incubated with LPS for 24h, and the relative mRNA expression of M1 cytokines between control, LPS, kaempferol (20, 40, 100 μM) group were detected by RT-PCR. (C) The ratio of M1 and M2 macrophages between control, LPS and kaempferol (40 μM) group were detected by flow cytometry. CD11b⁺ cells were recognized as macrophages, within CD80⁺ cells were recognized as M1 macrophages and CD206⁺ cells were recognized as M2 macrophages. One-way ANOVA was used and values are shown as means ± SD, n = 3–4 for each group. **p < 0.01 vs control group; +p < 0.05, ++p < 0.01 vs LPS group.

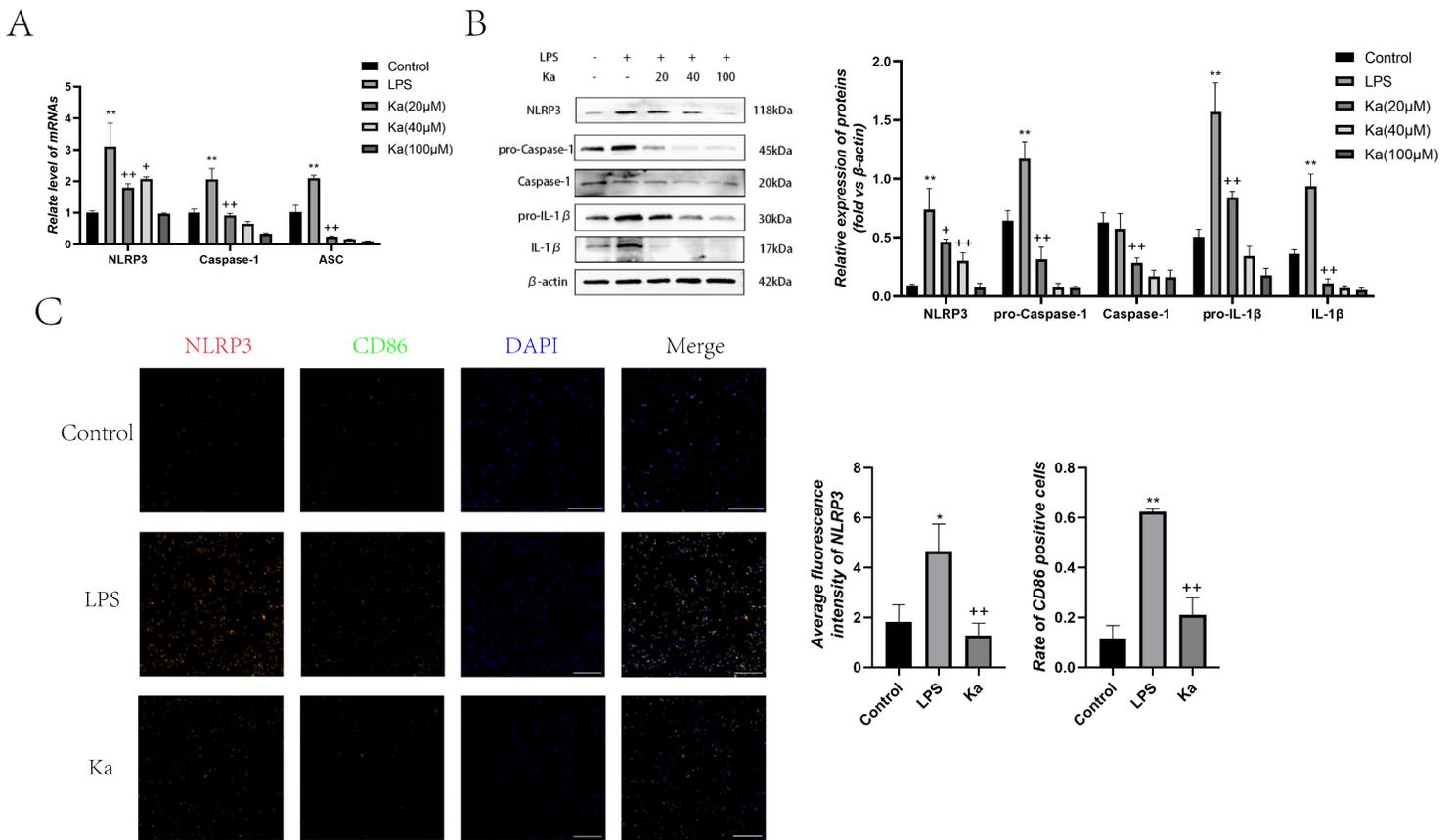


Figure 5

Kaempferol inhibited the activation of NLRP3 inflammasome in THP-1 derived macrophages. (A) The relative mRNA expression of NLRP3, Caspase-1, IL-1 β between control, LPS and kaempferol (20, 40, 100 μ M) group. (B) The relative protein level of NLRP3 inflammasome between control, LPS and kaempferol (20, 40, 100 μ M) group were detected by western blot analysis. (C) The relative expression of NLRP3 protein (red) and CD86 (green) positive cells between control, LPS and kaempferol (40 μ M) group were test by fluorescence microscopy. Scale bar, 200 μ m. One-way ANOVA was used and values are shown as means \pm SD, n = 3–4 for each group. * p < 0.05, ** p < 0.01 vs control group; + p < 0.05, ++ p < 0.01 vs LPS group.

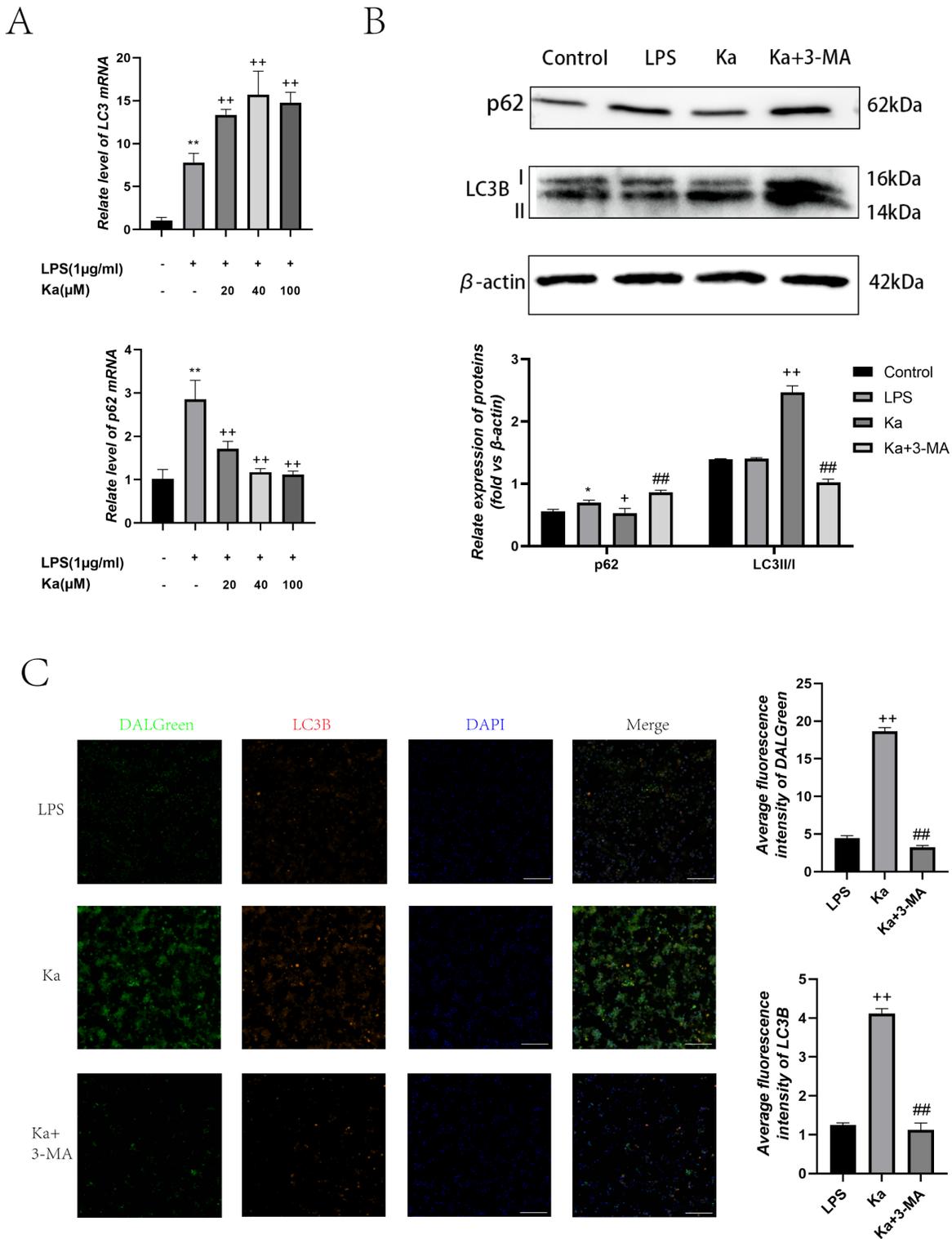


Figure 6

Kaempferol promoted autophagy in THP-1 derived macrophages. (A) The relative mRNA expression of LC3 and p62 between control, LPS and kaempferol (20, 40, 100µM) group. (B) In kaempferol+3-MA group, 3-MA (5mM) was added into the medium 30 minutes in advance, followed by LPS and kaempferol (40µM). The relative protein level of LC3 and p62 were detected by western blot. (C) DALGreen was transfected into macrophages before treatment according to the instruction. The relative fluorescence

intensity of LC3B (red) and DALGreen (green) between LPS, kaempferol (40 μ M) and kaempferol+3-MA group were detected by fluorescence microscopy. Scale bar, 200 μ m. One-way ANOVA was used and values are shown as means \pm SD, n = 3–4 for each group. *p<0.05, **p < 0.01 vs control group; +p < 0.05, ++p < 0.01 vs LPS group; ##p < 0.01 vs kaempferol group.

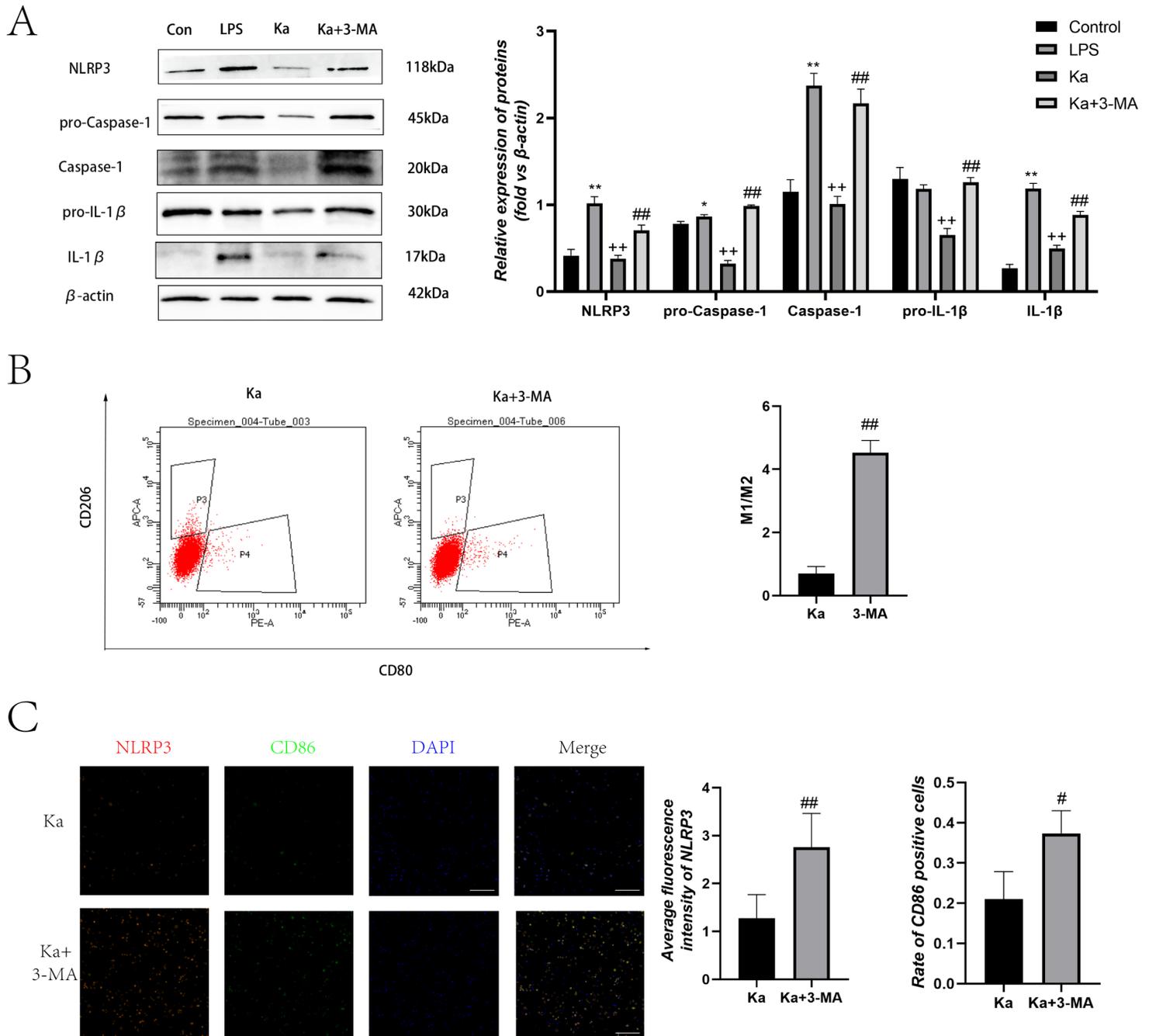


Figure 7

Autophagy inhibitor blocked the effect of kaempferol on NLRP3 inflammasome and macrophage polarization. (A) The relative protein level of NLRP3 inflammasome between control, LPS, kaempferol (40 μ M) and kaempferol+3-MA group were detected by western blot analysis. (B) M1/M2 between kaempferol (40 μ M) and kaempferol+3-MA group were detected by flow cytometry. (C) The rate of CD86 positive cells and relative level of NLRP3 were detected by fluorescence microscopy. Scale bar, 200 μ m.

One-way ANOVA was used and values are shown as means \pm SD, n = 3–4 for each group. **p < 0.01 vs control group; ++p < 0.01 vs LPS group; #p < 0.05, ##p < 0.01 vs kaempferol group.