

Dopamine Receptor 1 Treatment Promotes Epithelial Repair of Corneal Injury by Inhibiting NOD-Like Receptor Protein 3–Associated Inflammation

Licheng Li¹, Yang Yu¹, Zihao Zhuang¹, Qi Wu², Shu Lin^{2,3} and Jianmin Hu^{1,4}

¹Department of Ophthalmology, The Second Affiliated Hospital of Fujian Medical University, Engineering Research Centre of Assistive Technology for Visual Impairment, Fujian Province University, Quanzhou, Fujian Province, China

²Group of Neuroendocrinology, Garvan Institute of Medical Research, Sydney, Australia

³Centre of Neurological and Metabolic Research, The Second Affiliated Hospital of Fujian Medical University, Quanzhou, Fujian Province, China

⁴The School of Medical Technology and Engineering, Fujian Medical University, Fuzhou, Fujian Province, China

Correspondence: Jianmin Hu, Department of Ophthalmology, The Second Affiliated Hospital of Fujian Medical University, Engineering Research Centre of Assistive Technology for Visual Impairment, Fujian Province University, No. 34 North Zhongshan Road, Quanzhou, Fujian Province 362000, China; doctorhjm@163.com.

Shu Lin, Department of Ophthalmology, The Second Affiliated Hospital of Fujian Medical University, Engineering Research Centre of Assistive Technology for Visual Impairment, Fujian Province University, No. 34 North Zhongshan Road, Quanzhou, Fujian Province 362000, China; shulin1956@126.com.

LL and YY contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Received: August 23, 2023

Accepted: January 10, 2024

Published: January 31, 2024

Citation: Li L, Yu Y, Zhuang Z, Wu Q, Lin S, Hu J. Dopamine receptor 1 treatment promotes epithelial repair of corneal injury by inhibiting NOD-like receptor protein 3–associated inflammation. *Invest Ophthalmol Vis Sci*. 2024;65(1):49. <https://doi.org/10.1167/iovs.65.1.49>

PURPOSE. To elucidate the influence of dopamine receptor 1 (DRD1) on the proliferation of mouse corneal epithelial cells (MCECs) under inflammatory conditions.

METHODS. In vitro, immortalized MCECs (iMCECs) were treated with IL-1 β , with and without pcDNA3.1_DRD1. Primary MCECs (pMCECs) were exposed to IL-1 β , with and without DRD1 agonist (A68930). Cell proliferation was quantified using the Cell Counting Kit-8 (CCK-8) assay and immunofluorescence staining for Ki-67 and p63. Expression levels of NOD-like receptor protein 3 (NLRP3), IL-1 β , and IL-6 were assessed. To establish a corneal injury model in mice, a 2-mm superficial keratectomy was performed. Either 0.1% A68930 or PBS was topically administered three times daily to the injured eyes for up to 5 days post-injury. Immunofluorescence analysis was employed to evaluate the expression of Ki-67, p63, and CD45 in mouse corneas. Western blotting and real-time quantitative PCR were utilized for quantitative analysis of DRD1, NLRP3, IL-1 β , and IL-6 in mouse corneas. Corneal epithelial regeneration was monitored through fluorescein sodium staining for a duration of up to 5 days following the injury.

RESULTS. Overexpression of DRD1 and A68930 promoted MCEC proliferation and suppressed the expression of NLRP3, IL-1 β , and IL-6 in vitro. Topical application of the 0.1% A68930 following mechanical corneal injury in mice led to increased Ki-67 and p63 expression compared to PBS treatment. Furthermore, topical administration of the 0.1% A68930 reduced the expression of CD45, NLRP3, IL-1 β , and IL-6. Analysis with fluorescein sodium indicated accelerated corneal epithelial regeneration in the 0.1% A68930 treatment group.

CONCLUSIONS. DRD1 treatment counteracts NLRP3-associated inflammation and facilitates epithelial repair of corneal injury.

Keywords: corneal injury, inflammation, dopamine receptor 1, NOD-like receptor protein 3, damage repair

The corneal epithelium constitutes the outermost stratum of the cornea, serving as a crucial safeguard that upholds the intrinsic architecture of the cornea. Nevertheless, the corneal epithelium remains vulnerable to impairment stemming from its exposure to the encompassing milieu. Although endowed with self-regenerative capabilities due to the presence of limbal stem cells,¹ challenges or setbacks in the process of wound healing can manifest across an array of inflammatory and

metabolic ailments, including severe trauma and diabetes.^{2,3} Although inflammation plays a pivotal role in the reparative course of wound healing, persistent ocular inflammation coupled with the expression of pro-inflammatory cytokines can disrupt this intricate process, culminating in enduring epithelial deficiencies.⁴ Notwithstanding the diverse spectrum of therapeutic modalities available for addressing corneal epithelial anomalies, the risk of treatment insufficiency remains markedly high, potentially

precipitating corneal perforation and consequent loss of vision.⁵

Dopamine receptor 1 (DRD1) is a G-protein-coupled receptor that plays a pivotal role in a diverse range of physiological and pathological processes. Recent research has unveiled the capacity of DRD1 to influence inflammatory responses through its regulatory control over the activation of the NOD-like receptor protein 3 (NLRP3) signaling pathway, as well as its modulation of the expression of inflammatory mediators.⁶ In vitro, DRD1 has demonstrated its ability to protect microglial cells against impairment stemming from NLRP3-associated inflammation.⁷ Furthermore, in vivo, DRD1 has exhibited the potential to mitigate brain edema and ameliorate behavioral deficits by suppressing NLRP3-mediated inflammation in mice afflicted with intracerebral hemorrhage.⁸ Despite the acknowledged anti-inflammatory role of DRD1 in both in vitro and in vivo settings involving microglial cells, its potential therapeutic implications for the repair of corneal epithelial cells in inflamed and injured corneal tissues remain largely unexplored. Recent studies have reported that ropinirole can inhibit the expression of C-X-C motif chemokine ligand 1 (CXCL1) in gingival epithelial cells via dopamine receptors, leading to the suppression of neutrophil inflammation,⁹ but the specific signaling pathway associated with DRD1 in the regulation of NLRP3-driven inflammation in epithelial cells remains unexplored.

In the present study, our primary objective was to explore the impact of DRD1 on corneal epithelial cells under conditions of inflammation, both in vitro and in vivo. In our cell culture experiments, we enhanced the expression of DRD1 in pcDNA3.1 constructs, allowing us to precisely control receptor levels continuously. This strategy was effective in targeting the DRD1 mechanism. For our in vivo studies, we opted to use the DRD1 agonist A68930 to specifically activate the receptor. This method closely mimics physiological conditions for acute interventions and holds promise for potential future therapeutic applications. To bridge the gap between these two approaches, we conducted additional experiments involving the treatment of primary corneal epithelial cells in an inflammatory state with A68930.

Our collected data substantiate the assertion that overexpressed DRD1 and A68930 foster the proliferation of corneal epithelial cells, counteracting the suppressive influence exerted by inflammatory cytokines on these cellular entities. Furthermore, we have observed that overexpressed DRD1 and A68930 effectively mitigate the activation of inflammatory cells and hinder their production of NLRP3-associated inflammatory cytokines. To augment these findings, our in vivo observations employing a well-established sterile injury model of the mouse cornea underscore that topical application of A68930 effectively curbs ocular inflammation and notably accelerates the restoration process of corneal epithelial tissue.

MATERIALS AND METHODS

Animals

Seven-week-old male C57BL/6 wild-type mice (Shanghai Laboratory Animal Center, Shanghai, China) were used for these experiments.¹⁰ The protocol was approved by the Ethics Committee of The Second Affiliated Hospital of Fujian Medical University (2023215), and all animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Corneal Injury

Mice were anesthetized, and a 2-mm superficial keratectomy was performed.¹¹ Briefly, under a dissecting microscope, the central area of the cornea was demarcated with a 2-mm trephine. Corneal epithelium and basement membrane in the circular area were abraded using a hand-held miniature drill (RWD Life Science, San Diego, CA, USA). After the injury, the corneas were rinsed with sterile saline.

Application and Evaluation of DRD1 Agonists in Corneal Epithelial Repair

Five microliters of 0.1% DRD1 agonist (A68930; MedChem-Express, Monmouth Junction, NJ, USA) or PBS was applied topically to the injured eyes using a micropipette three times a day for up to 5 days post-injury. Corneal epithelial regeneration was evaluated by fluorescein sodium staining of the corneal surface to evaluate the area of epithelial defect.¹² Corneal fluorescein sodium staining was performed once a day for up to 5 days post-injury. Then, 0.5 μ L of 0.5% fluorescein sodium (Alcon, Geneva, Switzerland) was applied to the corneal surface using a micropipette. After 3 minutes, epithelial staining was scored in a masked fashion using slit lamp biomicroscopy (ChongQing KangHua, Chongqing, China) under cobalt blue light, and photographic images were taken. The area of the epithelial defect (green) was calculated using ImageJ (National Institutes of Health, Bethesda, MD, USA).

In Vitro Corneal Epithelial Cell Proliferation

Immortalized mouse corneal epithelial cells (iMCECs) were obtained from an immortalized cell line maintained by Immocell Biotechnology Co. (Xiamen, China). The iMCECs signify mouse corneal epithelial cells that have undergone immortalization, a process that allows cells to proliferate indefinitely. The iMCECs were cultured in MCEC immortalization medium (Immocell Biotechnology) with or without 1.4 μ g/mL of mouse pcDNA3.1_DRD1 or pcDNA3.1_vector (OBiO Technology, Shanghai, China). In addition, iMCECs were treated with 100 ng/mL of mouse recombinant IL-1 β (PeproTech, Cranbury, NJ, USA) with or without pcDNA3.1_DRD1 or pcDNA3.1_vector.

Primary mouse corneal epithelial cells (pMCECs) were also obtained from Immocell Biotechnology. The pMCECs represent primary cultures of mouse corneal epithelial cells. These cells are obtained directly from living tissue, maintaining their original characteristics. The pMCECs were cultured in MCEC medium (Immocell Biotechnology) with or without 10-, 20-, or 30- μ M A68930. In addition, pMCECs were treated with 100 ng/mL of mouse recombinant IL-1 β with or without 30- μ M A68930 or 1- μ M DRD1 antagonist (SCH23390; MedChemExpress).¹³

Here, the term MCECs refers to both iMCECs and pMCECs. MCECs were cultured for 24 hours, and the cultures were pulsed with Cell Counting Kit-8 (CCK-8) for the last 2 hours. MCEC proliferation was measured using the CCK-8 proliferation kit (Dalian Meilun Biotechnology, Dalian, China).

RNA Isolation and Real-Time Quantitative PCR

Total RNA was isolated using the RNA Extraction Kit (Takara, Shiga, Japan). Isolated RNA was reverse transcribed into cDNA using a Takara RT Reagent Kit. Then

TABLE. Primer List

GAPDH	Forward primer	AAATGGTGAAGGTCGGTGTGAAC
	Reverse primer	CAACAATCTCCACTTTGCCACTG
DRD1	Forward primer	GTAGCCATTATGATCGTCAC
	Reverse primer	GATCACAGACAGTGTCTTCAG
IL-1 β	Forward primer	GTGTGGATCCCAAGCAATAC
	Reverse primer	GGAACCTCTGCAGACTCAAAC
IL-6	Forward primer	AGTGGCTAAGGACCAAGA
	Reverse primer	GCCGAGTAGATCTCAAAGTG

real-time quantitative PCR (qPCR) was performed using Takara TB Green Premix Ex Taq and preformulated TaqMan primers for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), DRD1, IL-1 β , and IL-6 (Sangon Biotech, Shanghai, China). Primers are listed in the Table. The results were analyzed by the comparative threshold cycle method and normalized to GAPDH as an internal control.

Western Blotting

Total protein was extracted using the Protein Extraction Kit (Dalian Meilun Biotechnology). Extracted protein was loaded and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis. After being blocked with 5% skimmed milk for 2 hours, the membranes were incubated with rabbit anti-DRD1 (1:1000; Abcam, Cambridge, UK), rabbit anti-NLRP3 (1:1000; Abcam), and rabbit anti-GAPDH (1:10,000; Affinity Biosciences, Cincinnati, OH, USA) primary antibodies overnight at 4°C. The membranes were treated with horseradish peroxidase–labeled secondary antibody (Hangzhou Lianke Biology Technology, Zhejiang, China). Bands were visualized using enhanced chemiluminescence (ECL) detection reagents (Biosharp, Hefei, China). The relative density of protein was analyzed using the ImageJ software.

Immunofluorescence Staining

MCECs or cryosections of the whole eyeball were fixed with 4% paraformaldehyde (Biosharp) and blocked with 5% donkey serum (Beijing Solarbio Science & Technology, Beijing, China). Immunocytochemical staining was performed overnight with rabbit anti-NLRP3 (Proteintech, Rosemont, IL, USA) or PBS. Immunohistochemical staining with rabbit anti-CD45 (Proteintech), Ki-67 (Cell Signaling Technology, Danvers, MA, USA), p63 (Abcam), or PBS was performed overnight. MCECs or sections were then coupled to donkey anti-rabbit Alexa Fluor 488 or 594 for 2 hours. MCECs or slides were subsequently mounted with a 4',6-diamidino-2-phenylindole (DAPI) solution (Beijing Solarbio Science & Technology) and subjected to examination using a confocal microscope (Nikon, Tokyo, Japan). Imaging was performed with a 20 \times lens covering a scanning range specific to DAPI–fluorescein isothiocyanate (FITC)–tetramethylrhodamine isothiocyanate (TRITC) channels.

Statistical Analysis

Data are presented as the mean \pm SEM. Statistical significance was defined as $P < 0.05$. Statistical analyses were performed using the *t*-test for two groups or one-way ANOVA for multiple groups (GraphPad Software, Boston,

MA, USA). Samples sizes were estimated based on previous experimental studies on corneal injury and inflammation.

RESULTS

Overexpressed DRD1 Augments iMCEC Proliferation and Reverses IL-1 β –Mediated Inhibition

To investigate the impact of overexpressed DRD1 on the proliferation of iMCECs, several experiments were conducted. iMCECs were cultivated under various conditions, including MCEC immortalization medium supplemented with or without 1.4 μ g/mL of mouse pcDNA3.1_DRD1 or pcDNA3.1_vector. The assessment of iMCEC proliferation was performed using the CCK-8 assay. The results revealed a noteworthy increase in iMCEC proliferation among cells cultivated in a MCEC immortalization medium containing mouse pcDNA3.1_DRD1 as compared to those cultured in the MCEC immortalization medium alone (Fig. 1C). Subsequently, the focus shifted toward exploring the influence of overexpressed DRD1 on iMCEC proliferation within an inflammatory context. iMCECs were cultured in a MCEC immortalization medium with or without 100 ng/mL of IL-1 β alongside the presence or absence of pcDNA3.1_DRD1 or pcDNA3.1_vector. Despite the inhibitory effect of IL-1 β on iMCEC proliferation, the overexpression of DRD1 effectively counteracted the antiproliferative impact of IL-1 β (Fig. 1D). These in vitro findings collectively suggest that overexpression of DRD1 plays a role in promoting the proliferation of iMCECs under both normal physiological conditions and within an inflammatory milieu.

Overexpressed DRD1 Suppresses the Expression of NLRP3-Associated Inflammatory Cytokines

To investigate the potential role of overexpressed DRD1 in the regulation of the inflammatory response, we examined its impact on the expression of NLRP3-associated proinflammatory factors—namely, IL-1 β and IL-6. iMCECs were exposed to IL-1 β in the presence or absence of overexpressed DRD1 for a duration of 24 hours. Subsequently, the expression levels of NLRP3, IL-1 β , and IL-6 were assessed using immunofluorescence staining and real-time PCR techniques. Our findings revealed that, whereas IL-1 β prominently increased the expression of NLRP3, IL-1 β , and IL-6 in iMCECs, the overexpression of DRD1 significantly attenuated the expression of these proinflammatory factors (Figs. 2A–2D). These outcomes strongly indicate that overexpressed DRD1 exerts a suppressive effect on the expression of NLRP3-related inflammatory cytokines.

DRD1 Agonist Boosts pMCEC Proliferation and Reverses IL-1 β Inhibition Via the NLRP3 Signaling Pathway

The investigation into the impact of the DRD1 agonist A68930 on the proliferation of pMCECs involved a series of experiments conducted under various conditions. Cultivation of pMCECs in MCEC medium supplemented with 10-, 20-, or 30- μ M A68930 revealed a significant increase in cell proliferation at 30 μ M compared to cells cultured in MCEC medium alone (Fig. 3A), as assessed by the CCK-8 assay.

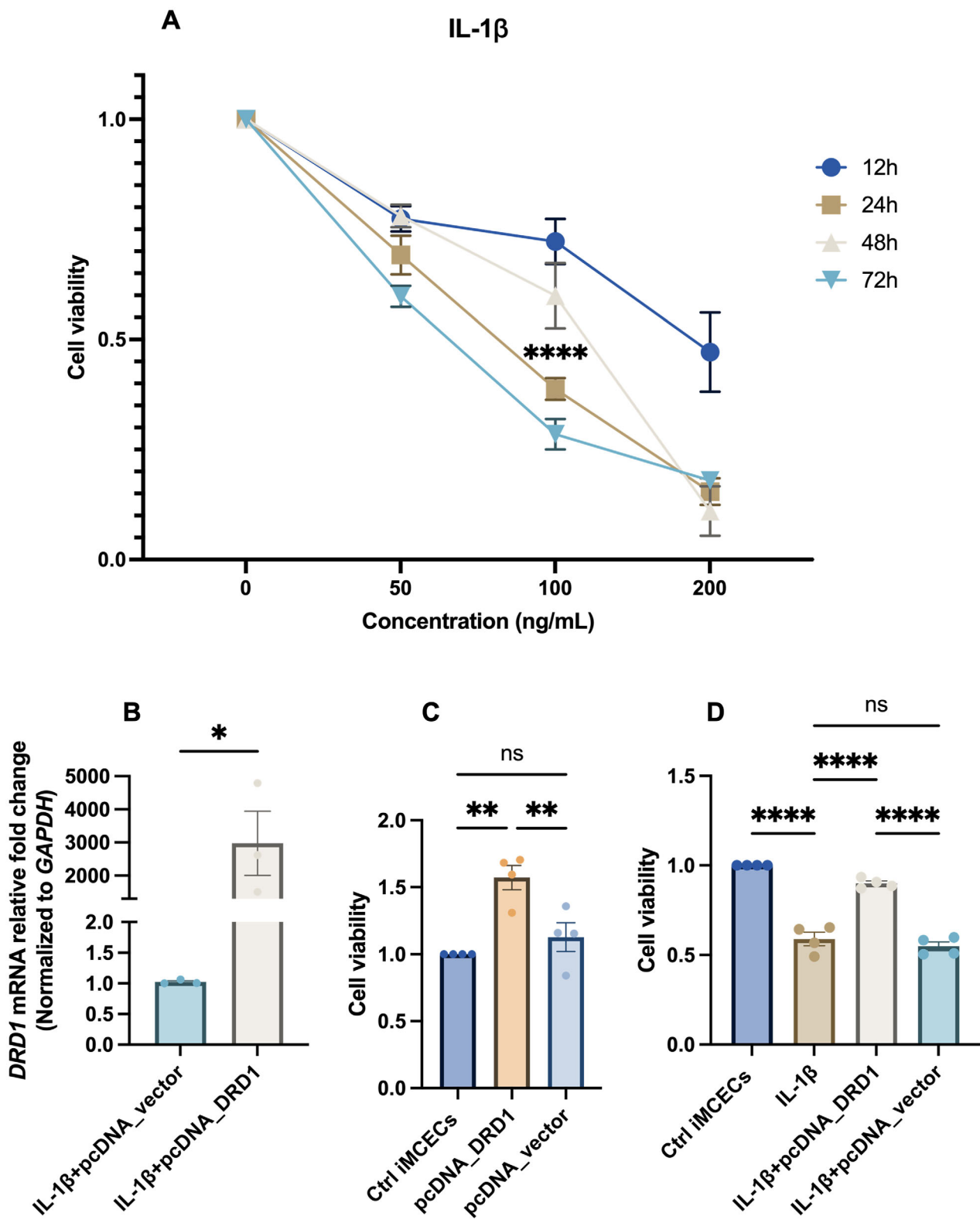


FIGURE 1. Cell viability after exposure to IL-1 β . Control iMCECs had no additional experimental interventions. **(A)** The effect of different concentrations of IL-1 β (0, 50, 100, 200 ng/mL) at different times (12, 24, 48, 72 hours) of intervention on the cell viability of iMCECs. **(B)** DRD1 was successfully overexpressed in iMCECs by pcDNA_DRD1. **(C)** Quantification analysis of iMCEC proliferation during homeostatic conditions via CCK-8 assay. **(D)** Quantification analysis of iMCEC proliferation in an inflammatory environment via CCK-8 assay. Data are represented as mean \pm SEM. Experiments were repeated three or four times, and data in each group are from triplicate or quadruplicate wells. * P < 0.05, ** P < 0.01, *** P < 0.005, **** P < 0.001; ns, not significant.

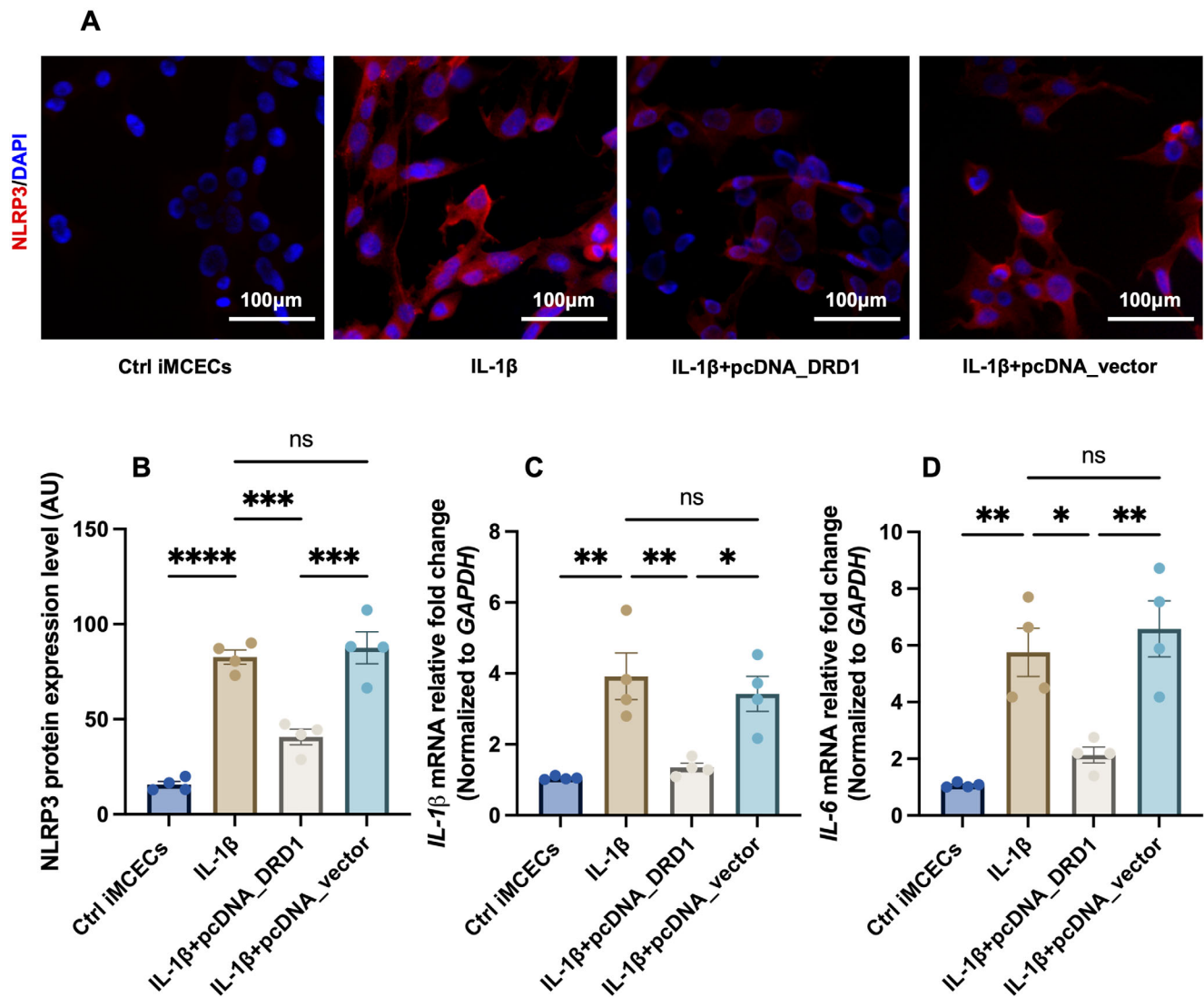


FIGURE 2. Overexpressed DRD1 inhibited NLRP3-related inflammatory factors in iMCECs. (A) Representative confocal microscopic images of the NLRP3⁺ iMCECs. Scale bars: 100 μ m. (B) Quantification analysis of NLRP3 protein expression via immunofluorescence staining. (C) Quantification analysis of IL-1 β mRNA expression via real-time qPCR. (D) Quantification analysis of IL-6 mRNA expression via real-time qPCR. Data are represented as mean \pm SEM. Experiments were repeated four times, and data in each group are from quadruplicate wells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$; ns, not significant.

Subsequent experiments explored the influence of 30 μ M A68930 on pMCEC proliferation in an inflammatory context. pMCECs were cultured in MCEC medium with or without 100 ng/mL IL-1 β , with the presence or absence of 30- μ M A68930 or 1- μ M SCH23390. Despite the inhibitory effect of IL-1 β on pMCEC proliferation, 30- μ M A68930 effectively counteracted this antiproliferative impact. This was evidenced by increased expression of the proliferative markers Ki-67 and p63 within DRD1 agonist-treated pMCECs (Figs. 3D–3F).

To explore the potential regulatory role of 30 μ M A68930 in the inflammatory response, its impact on NLRP3 expression was examined. pMCECs were exposed to IL-1 β in the presence or absence of 30- μ M A68930 for 24 hours. The results demonstrated that, although IL-1 β increased NLRP3 expression, 30- μ M A68930 significantly attenuated it (Figs. 3B, 3C).

The in vitro findings collectively suggest that 30- μ M A68930 promotes pMCEC proliferation under both normal

physiological conditions and in an inflammatory milieu. These outcomes strongly indicate that 30- μ M A68930 exerts a suppressive effect on NLRP3 expression.

DRD1 Agonist Enhances Epithelial Cell Proliferation in an In Vivo Model of Corneal Injury

To investigate the impact of treatment with the DRD1 agonist A68930 on the proliferation of living corneal epithelial cells, we employed a well-established murine model of corneal injury. Mechanical injury was induced in mice, followed by the administration of either topical 0.1% A68930 or PBS to the injured corneas three times daily for a duration of 5 days. Uninjured corneas and injured corneas treated with PBS were employed as comparative controls. Corneal tissues were collected on the first day following injury and

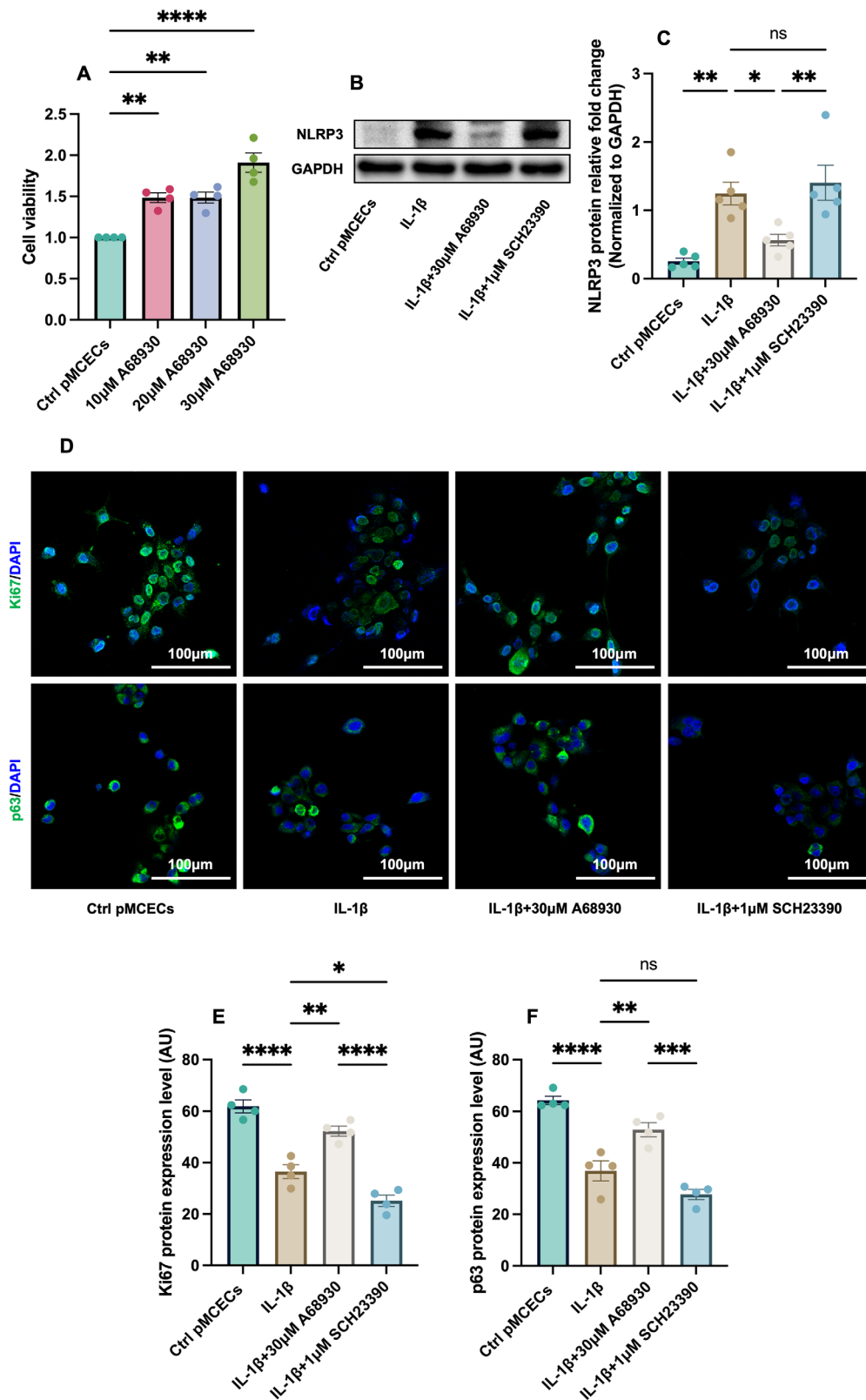


FIGURE 3. DRD1 agonist promoted pMCEC proliferation and inhibited expression of NLRP3. The DRD1 agonist was A68930, and the DRD1 antagonist was SCH23390. (A) The effect of different concentrations of A68930 (0, 10, 20, 30 μM) on the cell viability of pMCECs. (B) Representative immunoblot images of the expression of NLRP3 and GAPDH protein. (C) Quantification analysis of NLRP3 protein expression via western blotting. (D) Representative confocal microscopic images of the Ki-67⁺ and p63⁺ pMCECs. Scale bars: 100 μm. (E) Quantification analysis of Ki-67⁺ pMCECs via immunofluorescent staining. (F) Quantification analysis of p63⁺ pMCECs via immunofluorescent staining. Data are represented as mean ± SEM. Experiments were repeated four or five times, and data in each group are from quadruplicate or quintuplicate wells. **P* < 0.05, ***P* < 0.01, ****P* < 0.005, *****P* < 0.001; ns, not significant.

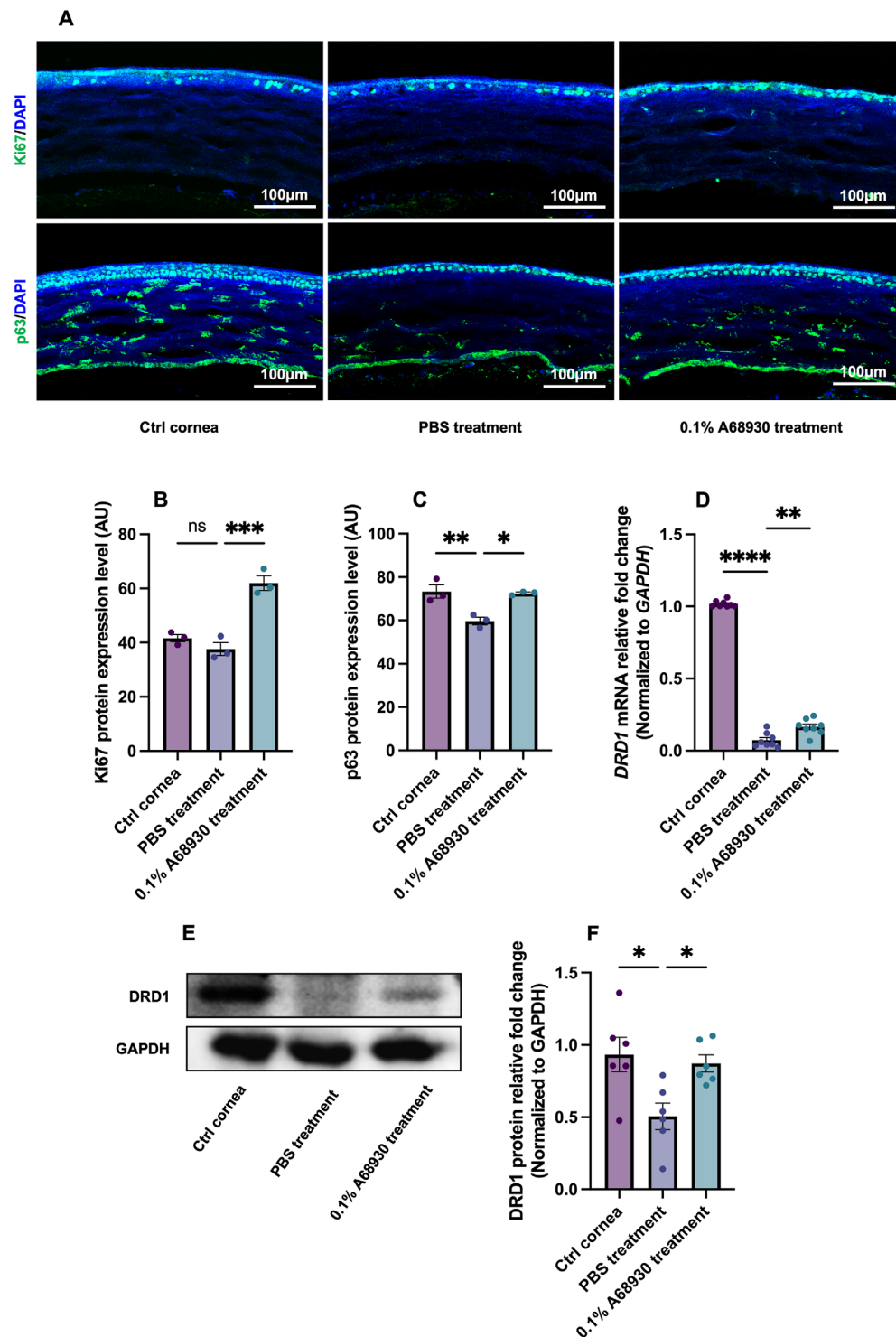


FIGURE 4. Administration of 0.1% A68930 promoted MCEC proliferation and expression of DRD1 in vivo. (A) Representative confocal microscopic images of the Ki-67⁺ and p63⁺ cells. Scale bars: 100 μ m. (B) Quantification analysis of Ki-67⁺ cells in corneas via immunofluorescent staining. (C) Quantification analysis of p63⁺ cells via immunofluorescent staining. (D) Quantification analysis of DRD1 mRNA expression via real-time qPCR. (E) Representative immunoblot images of the expression of DRD1 and GAPDH protein. (F) Quantification analysis of DRD1 protein expression via western blotting. Data are represented as mean \pm SEM. Experiments were repeated three to eight times. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$; ns, not significant.

subsequently utilized for the evaluation of corneal epithelial cell proliferation. Immunofluorescent staining targeting Ki-67 and p63, recognized markers of cellular proliferation, exhibited heightened expression within 0.1% A68930-

treated corneas when compared with the control counterparts (Fig. 4A). Quantitative analysis demonstrated a noteworthy increase in the quantity of Ki-67⁺ cells and p63⁺ cells within the corneas treated with 0.1% A68930

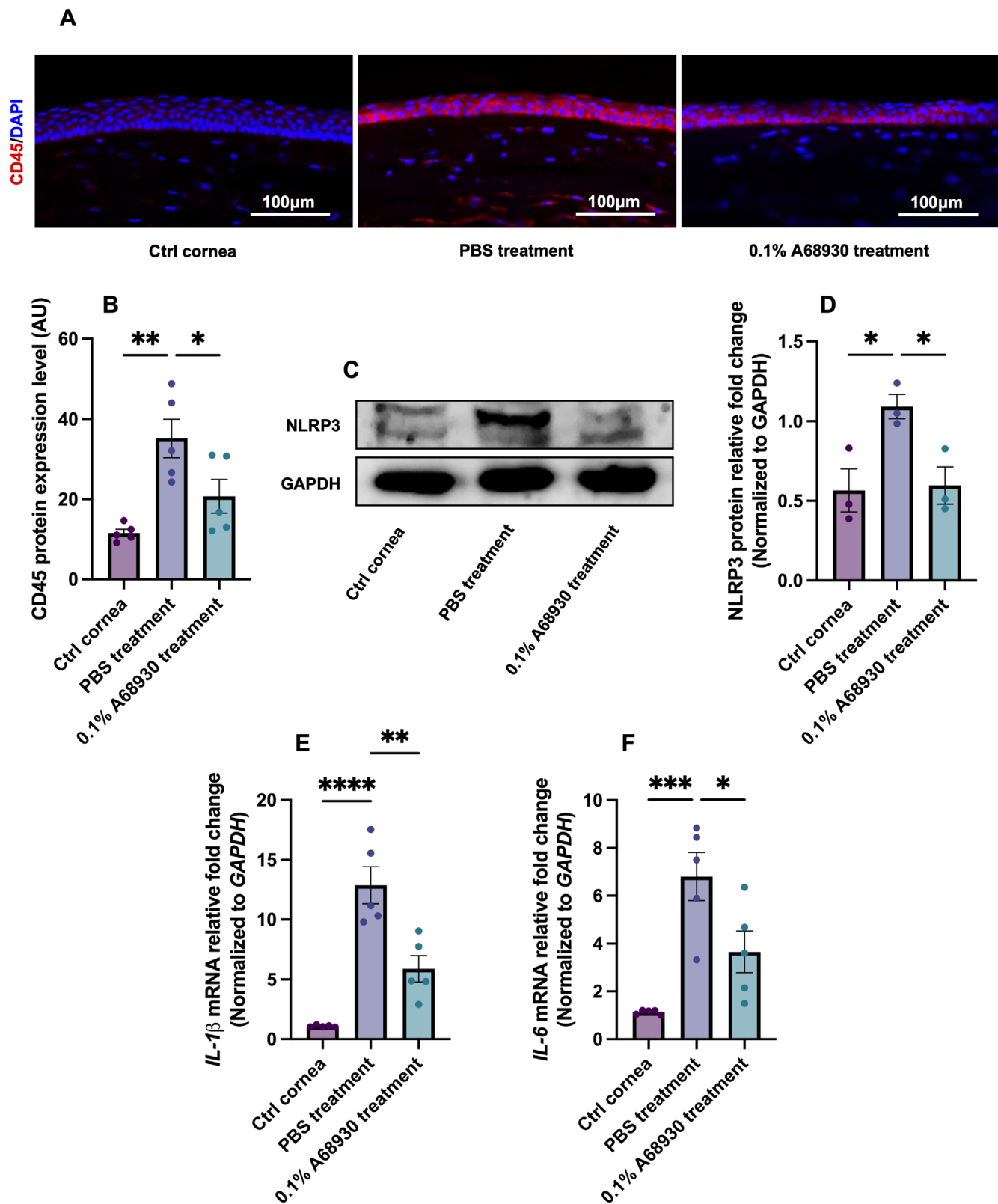


FIGURE 5. Administration of 0.1% A68930 inhibited ocular inflammation and expression of NLRP3-associated inflammatory factors in vivo. Control corneas had no additional experimental interventions. (A) Representative confocal microscopic images of the CD45⁺ cells. Scale bars: 100 μ m. (B) Quantification analysis of CD45⁺ cells in corneas. (C) Representative immunoblot images of the NLRP3 and GAPDH protein. (D) Quantification analysis of NLRP3 protein expression via western blotting. (E) Quantification analysis of IL-1 β mRNA expression via real-time qPCR. (F) Quantification analysis of IL-6 mRNA expression via real-time qPCR. Data are represented as mean \pm SEM. Experiments were repeated three to five times. * P < 0.05, ** P < 0.01, *** P < 0.005, **** P < 0.001; ns, not significant.

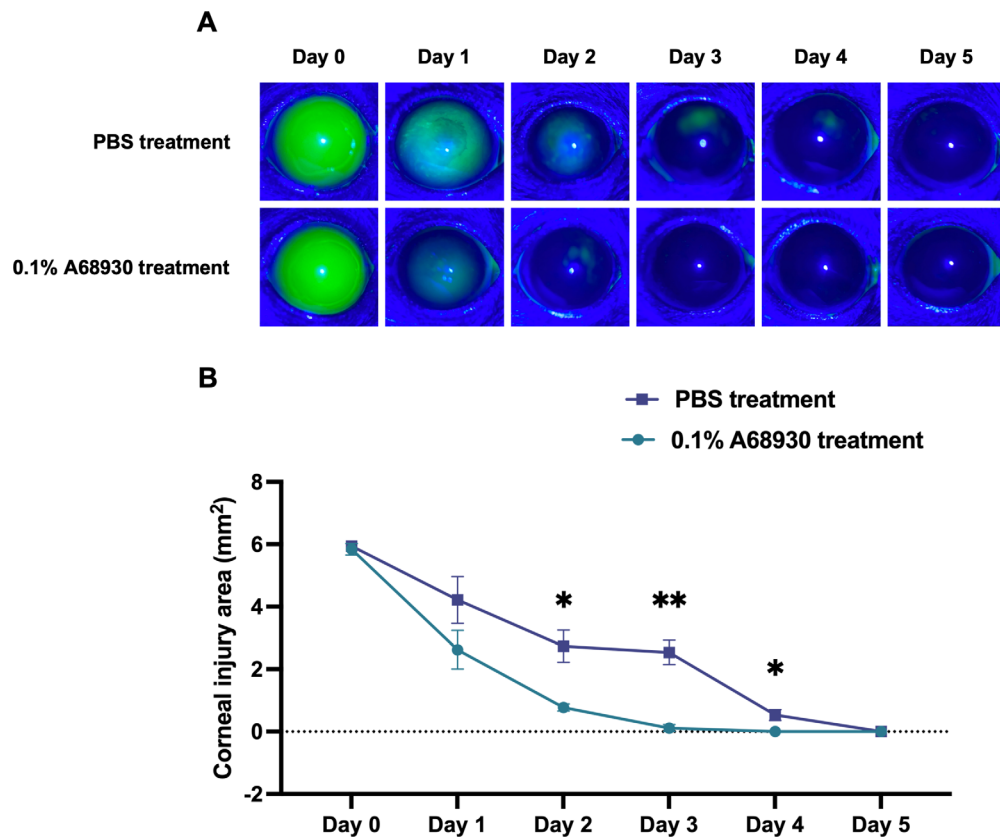


FIGURE 6. Administration of 0.1% A68930 promoted epithelial wound healing in mice with corneal injury. (A) Representative images of the epithelial defect in wounded corneas of PBS and 0.1% A68930 treatment (fluorescein sodium staining of corneal surface). (B) Quantification analysis of re-epithelialization in PBS and 0.1% A68930-treated mice corneas. Data are represented as mean \pm SEM. Experiments were repeated three times. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$; ns, not significant.

(Figs. 4B, 4C). Furthermore, corneas treated with 0.1% A68930 displayed escalated levels of DRD1 mRNA and protein compared with the PBS-treated control corneas (Figs. 4D–4F).

DRD1 Agonist Mitigates Ocular Inflammation and Enhances Epithelial Wound Healing in Murine Corneal Injury

Based on the observed anti-inflammatory properties of DRD1 in vitro, we assessed the impact of topically administered 0.1% A68930 on corneal inflammation following epithelial injury induction. On the first day post-injury, corneas were harvested, and leukocyte infiltration in the cornea was assessed by staining the corneas with the pan-leukocyte marker CD45. Confocal microscopic images of immunostained corneas revealed an elevated count of CD45⁺ cells in injured corneas treated with PBS compared to uninjured corneas. However, treatment with 0.1% A68930 significantly suppressed CD45⁺ cell infiltration in the injured corneas (Figs. 5A, 5B). To further explore the inhibitory effect of 0.1% A68930 on corneal inflammation, the expression of NLRP3, IL-1 β , and IL-6 was quantified using western blotting or real-time PCR. The expression of NLRP3, IL-1 β , and IL-6 was notably elevated after injury when compared with the control corneas. Nonetheless, treatment with 0.1% A68930 led to a substantial reduction in the expression of these inflammatory cytokines in the injured corneas (Figs. 5C–5F).

Finally, to investigate the potential impact of 0.1% A68930 on corneal wound repair, epithelial healing was monitored over a span of 5 days following corneal injury induction by utilizing corneal fluorescein sodium staining. We observed rapid healing of the corneal epithelium in the eyes treated with 0.1% A68930, as evidenced by the reduced area of fluorescein sodium uptake (green). Complete epithelial closure was achieved within 4 days in mice treated with 0.1% A68930. In contrast, control mice treated with PBS exhibited punctate fluorescein sodium staining of the epithelium up to day 4 post-injury (Fig. 6A). A quantitative evaluation of the extent of epithelial injury indicated a significantly smaller area of non-healed epithelium in the eyes treated with 0.1% A68930 on days 1, 2, 3, and 4 post-injury compared to the PBS-treated controls (Fig. 6B). Collectively, these findings suggest that treatment with the DRD1 agonist inhibits the infiltration of inflammatory cells into the tissue, suppresses their expression of pro-inflammatory cytokines, and enhances the repair of the corneal epithelium in cases of eye injury.

DISCUSSION

Insufficient corneal wound healing has the potential to result in diminished corneal transparency, thereby impacting visual acuity. Inflammatory responses have garnered significant attention in recent times for their role in influencing the process of corneal wound healing.^{11,14,15} In our investigation, we illustrated that DRD1 facilitates the prolif-

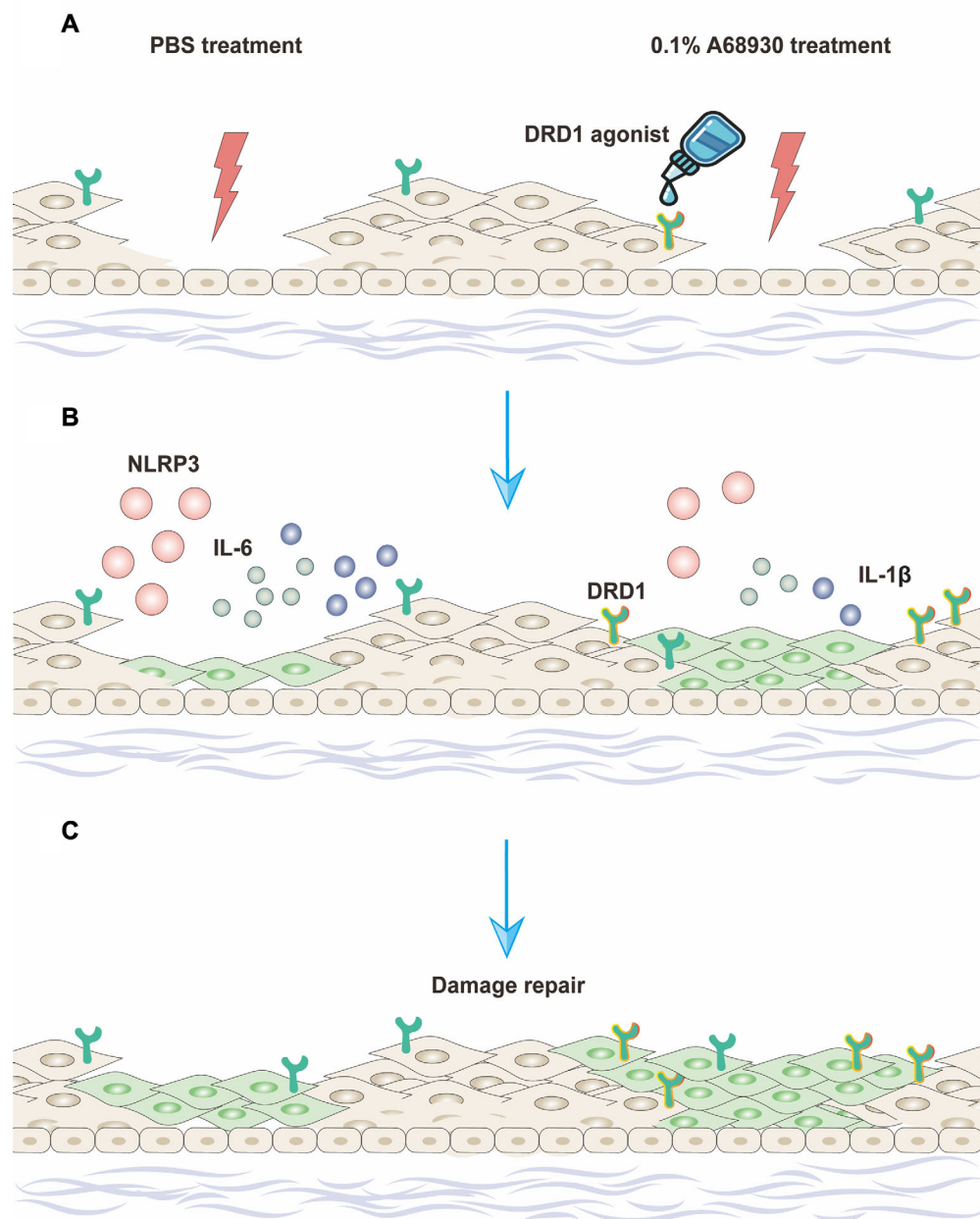


FIGURE 7. Administration of 0.1% A68930 promoted the corneal wound healing process. (A) The PBS treatment group and the 0.1% A68930 treatment group were treated with the same corneal injury. (B) After injury, expression of DRD1 was downregulated and NLRP3, IL-1 β , and IL-6 levels were upregulated. (C) The 0.1% A68930 promoted corneal wound healing by upregulation of DRD1 and downregulation of NLRP3-associated inflammatory factors.

eration of MCECs within an inflammatory milieu while also impeding the activation and operation of inflammatory cells under *in vitro* conditions. Furthermore, we ascertained that the application of a topical DRD1 agonist in an *in vivo* model of corneal injury effectively suppresses ocular inflammation and yields substantial enhancement in corneal epithelial regeneration. This improvement is underscored by elevated expressions of the proliferative markers Ki-67 and p63 within DRD1 agonist-treated mice.

Although inflammation is essential for proper wound healing, excessive and prolonged inflammation may lead to delayed epithelial recovery and the development of corneal scarring, ulceration, or perforations.^{16,17} In cases of ocular inflammation, there is an upregulation in the expression of IL-1 β that leads to the initiation of apoptosis or pyroptosis in corneal epithelial cells. This, in turn, hampers the process of wound healing in traumatized ocular tissues.^{18,19} Prior investigations into the interplay between DRD1 and

inflammation have revealed that DRD1 functions as an anti-inflammatory agent by impeding activation of the NLRP3 inflammasome and the subsequent release of inflammatory cytokines, including caspase-1 and IL-1 β .⁶ Recent studies have elucidated the anti-inflammatory effects of DRD1 in conditions such as cerebral hemorrhage, acute kidney injury, and cardiac injury.^{8,20,21} A study has documented that the engagement of DRD1 through A68930 yielded improvements in cerebral edema among mice with cerebral hemorrhage. This effect was achieved via the suppression of NLRP3, caspase-1, and IL-1 β .⁸ An additional study revealed that A68930 exhibited substantial enhancement in renal functionality, achieved through the reduction of macrophage and T-cell infiltration, dampening the activation of the NLRP3 inflammasome and curtailing the expression of pro-inflammatory cytokines (TNF- α , IL-6, IL-1 β).²⁰ Although the stimulation of DRD1 and DRD2 through pergolide has been shown to stimulate corneal nerve regeneration in a murine corneal scratch model,²² the impact of DRD1 on NLRP3-mediated inflammation in cases of corneal epithelial injury remains to be comprehensively elucidated.

In our investigation, we observed that the overexpression of DRD1 not only augmented the proliferation of MCECs during homeostatic conditions but also effectively reversed the growth-suppressing impact of IL-1 β in vitro. This finding indicates that DRD1 actively counteracts the inhibitory influence of an inflammatory microenvironment on corneal epithelium. Furthermore, our in vivo findings furnish novel evidence demonstrating that the topical application of a DRD1 agonist (in this case, A68930) subsequent to corneal injury markedly diminishes the infiltration of CD45⁺ inflammatory cells within the cornea. Elevated infiltration of CD45⁺ inflammatory cells manifests as a pseudo-epithelial cell layer in the anterior stroma of injured corneas treated with PBS. This formation of an inflammatory cell layer impedes the migration of epithelial cells toward the site of injury, consequently retarding wound repair. Remarkably, treatment with the DRD1 agonist substantially curbed CD45⁺ cell infiltration and development of the inflammatory cell layer in the injured cornea. Additionally, the expression levels of NLRP3, IL-1 β , and IL-6 inflammatory factors in the wounded cornea exhibited a significant reduction following treatment with the DRD1 agonist. These outcomes corroborate earlier studies suggesting that DRD1 exerts a repressive influence on NLRP3-associated inflammatory responses within acute injury models.^{8,20} Furthermore, we noted a heightened expression of DRD1 within corneas treated with the DRD1 agonist, suggesting that the augmented DRD1 signaling may explain the heightened immunosuppressive effects and heightened epithelial cell proliferation mediated by the DRD1 agonist during the process of corneal wound healing.

In summary, this study highlights that the topical application of a DRD1 agonist in corneal injuries has the potential to enhance epithelial cell proliferation, suppress NLRP3-associated inflammation in the cornea, and significantly expedite the healing of corneal epithelial wounds through its capacity to counteract the inhibitory influence of an inflammatory milieu (Fig. 7). Our findings provide fresh insights into the immunomodulatory and regenerative roles of the DRD1 agonist, offering a conceptual foundation for the development of innovative therapies centered around DRD1 for addressing persistent corneal epithelial defects. Nonetheless, it is important to note that the present study

only monitored the impact of 0.1% A68930 eye drops on the local cornea and did not have data to assess its effects on the entire body. Therefore, future investigations to determine whether or not 0.1% A68930 eye drops exert significant systemic effects are essential for assessing its potential clinical applicability. In addition, the present study did not delve into the interplay between DRD1 and NLRP3 within corneal epithelial cells and immune cells, such as macrophages and T cells, at the cellular level. Recent investigations have revealed that nerve reflexes, orchestrated by the hypothalamus, contribute to corneal inflammation.²³ DRD1, being a prevalent neuroreceptor, likely plays a role in the inflammatory feedback loop within the central axis in the context of corneal injury. Delving into these intricate mechanisms holds promise for future explorations within the realm of corneal injury research.

Emerging research encompasses a range of innovative approaches and interventions designed to enhance corneal wound healing and address associated complications. The discussed treatments, including applied insulin-like growth factor 1 (IGF-1) modRNA-engineered adipose-derived mesenchymal stem cells (ADSCs^{modIGF1}),²⁴ extracellular vesicles derived from ADSCs (ADSC-EVs),²⁵ electrospun scaffolds functionalized with ROS-scavenging hydrogels (RH-ESs),²⁶ highly stable fibronectin-mimetic-peptide-based supramolecular hydrogels (Nap-FFPHSRN hydrogels),²⁷ and perfluorodecalin-based supersaturated oxygen emulsion (SSOE),²⁸ demonstrate significant potential in improving both the morphology and function of the cornea. For example, ADSC^{modIGF1} treatment demonstrates remarkable advancements in corneal recovery, surpassing simple ADSCs and IGF-1 protein eyedrops, with positive impacts on epithelial, limbal, stromal, angiogenic, lymphangiogenic, and nerve repair aspects.²⁴ ADSC-EVs effectively promote diabetic corneal epithelial wound healing via the nerve growth factor (NGF)/TrkA pathway, particularly involving dendritic cells.²⁵ The application of RH-ESs in a rat corneal alkali burn model yields significant benefits, including reduced inflammation, downregulation of inflammation-related genes, suppression of vascularization and scarring, and accelerated corneal wound healing.²⁶ The Nap-FFPHSRN hydrogels, designed to mimic bioactive proteins, demonstrate prolonged precorneal retention and enhanced therapeutic effects, promoting corneal re-epithelialization in a scrape model.²⁷ Future research can explore the use of the aforementioned carriers to package and transport A68930, potentially developing eye drops with enhanced performance.

Acknowledgments

The authors thank Jianqing Zheng and Jin Chen for their excellent technical support.

Supported in part by a grant from the Science and Technology Bureau of Quanzhou (2020CT003).

Disclosure: **L. Li**, None; **Y. Yu**, None; **Z. Zhuang**, None; **Q. Wu**, None; **S. Lin**, None; **J. Hu**, None

References

- Altshuler A, Amitai-Lange A, Tarazi N, et al. Discrete limbal epithelial stem cell populations mediate corneal homeostasis and wound healing. *Cell Stem Cell*. 2021;28:1248–1261.e8.

2. Yu FX, Lee PSY, Yang L, et al. The impact of sensory neuropathy and inflammation on epithelial wound healing in diabetic corneas. *Prog Retin Eye Res.* 2022;89:101039.
3. Chouhan G, Moakes RJA, Esmaceli M, et al. A self-healing hydrogel eye drop for the sustained delivery of decorin to prevent corneal scarring. *Biomaterials.* 2019;210:41–50.
4. Ma X, Wang Q, Song F, et al. Corneal epithelial injury-induced norepinephrine promotes *Pseudomonas aeruginosa* keratitis. *Exp Eye Res.* 2020;195:108048.
5. Hos D, Matthaie M, Bock F, et al. Immune reactions after modern lamellar (DALK, DSAEK, DMEK) versus conventional penetrating corneal transplantation. *Prog Retin Eye Res.* 2019;73:100768.
6. Yan Y, Jiang W, Liu L, et al. Dopamine controls systemic inflammation through inhibition of NLRP3 inflammasome. *Cell.* 2015;160:62–73.
7. Pike AF, Longhena F, Faustini G, et al. Dopamine signaling modulates microglial NLRP3 inflammasome activation: implications for Parkinson's disease. *J Neuroinflammation.* 2022;19:50.
8. Wang T, Nowrangi D, Yu L, et al. Activation of dopamine D1 receptor decreased NLRP3-mediated inflammation in intracerebral hemorrhage mice. *J Neuroinflammation.* 2018;15:2.
9. Iozaki Y, Sato T, Takagi R, et al. Ropinirole inhibits inflammatory cytokine production in gingival epithelial cells and suppresses alveolar bone loss in an experimental rat model of periodontitis. *Exp Ther Med.* 2023;25:78.
10. Zhu L, Wang L, Liu D, et al. Single-cell transcriptomics implicates the FEZ1-DKK1 axis in the regulation of corneal epithelial cell proliferation and senescence. *Cell Prolif.* 2023;56:e13433.
11. Wu M, Downie LE, Hill LJ, Chinnery HR. The effect of topical decorin on temporal changes to corneal immune cells after epithelial abrasion. *J Neuroinflammation.* 2022;19:90.
12. Lan X, Zhang W, Zhu J, et al. dsRNA induced IFN β -MMP13 axis drives corneal wound healing. *Invest Ophthalmol Vis Sci.* 2022;63:14.
13. Wang YH, Lv HN, Cui QH, Tu PF, Jiang Y, Zeng KW. Isosibiricin inhibits microglial activation by targeting the dopamine D1/D2 receptor-dependent NLRP3/caspase-1 inflammasome pathway. *Acta Pharmacol Sin.* 2020;41:173–180.
14. Omoto M, Suri K, Amouzegar A, et al. Hepatocyte growth factor suppresses inflammation and promotes epithelium repair in corneal injury. *Mol Ther.* 2017;25:1881–1888.
15. Balne PK, Gupta S, Landon KM, et al. Characterization of C-X-C chemokine receptor type 5 in the cornea and role in the inflammatory response after corneal injury. *Exp Eye Res.* 2023;226:109312.
16. Wu M, Hill LJ, Downie LE, Chinnery HR. Neuroimmune crosstalk in the cornea: the role of immune cells in corneal nerve maintenance during homeostasis and inflammation. *Prog Retin Eye Res.* 2022;91:101105.
17. Kiritoshi S, Soma T. Corneal perforation secondary to gonococcal keratoconjunctivitis. *CMAJ.* 2020;192:E1361.
18. Li J, Yang K, Pan X, et al. Long noncoding RNA MIAT regulates hyperosmotic stress-induced corneal epithelial cell injury via inhibiting the caspase-1-dependent pyroptosis and apoptosis in dry eye disease. *J Inflamm Res.* 2022;15:3269–3283.
19. Zhao W, Yang H, Lyu L, et al. GSDMD, an executor of pyroptosis, is involved in IL-1 β secretion in *Aspergillus fumigatus* keratitis. *Exp Eye Res.* 2021;202:108375.
20. Cao JY, Zhou LT, Li ZL, Yang Y, Liu BC, Liu H. Dopamine D1 receptor agonist A68930 attenuates acute kidney injury by inhibiting NLRP3 inflammasome activation. *J Pharmacol Sci.* 2020;143:226–233.
21. Liu J, Jin Y, Wang B, Wang Y, Zuo S, Zhang J. Dopamine D1 receptor alleviates doxorubicin-induced cardiac injury by inhibiting NLRP3 inflammasome. *Biochem Biophys Res Commun.* 2021;561:7–13.
22. Zhang X, Muddana S, Kumar SR, et al. Topical pergolide enhance corneal nerve regrowth following induced corneal abrasion. *Invest Ophthalmol Vis Sci.* 2020;61:4.
23. Lasagni Vitar RM, Fonteyne P, Chaabane L, Rama P, Ferrari G. A hypothalamic-controlled neural reflex promotes corneal inflammation. *Invest Ophthalmol Vis Sci.* 2021;62:21.
24. Yu F, Gong D, Yan D, et al. Enhanced adipose-derived stem cells with IGF-1-modified mRNA promote wound healing following corneal injury. *Mol Ther.* 2023;31:2454–2471.
25. Wang G, Zeng L, Gong C, Gong X, Zhu T, Zhu Y. Extracellular vesicles derived from mouse adipose-derived mesenchymal stem cells promote diabetic corneal epithelial wound healing through NGF/TrkA pathway activation involving dendritic cells. *Exp Eye Res.* 2023;231:109484.
26. Shi X, Zhou T, Huang S, et al. An electrospun scaffold functionalized with a ROS-scavenging hydrogel stimulates ocular wound healing. *Acta Biomater.* 2023;158:266–280.
27. Hu Y, Shi H, Ma X, et al. Highly stable fibronectin-mimetic-peptide-based supramolecular hydrogel to accelerate corneal wound healing. *Acta Biomater.* 2023;159:128–139.
28. Li S, Pang K, Zhu S, Pate K, Yin J. Perfluorodecalin-based oxygenated emulsion as a topical treatment for chemical burn to the eye. *Nat Commun.* 2022;13:7371.