Neuroprotective role of epigallocatechin-3-gallate in acute glaucoma via the nuclear factor-κB signalling pathway

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Abstract. Glaucoma is a disease involving impaired visual function accompanied by degeneration and necrosis of the optic nerve. Epigallocatechin-3-gallate (EGCG) exerts a neuroprotective effect against the degeneration of retinal ganglion cells. However, whether EGCG can relieve glaucoma and the possible mechanisms remain unclear. In order to determine the function of EGCG in glaucoma, an acute glaucoma rat model was established. Optic neuropathology was examined by haematoxylin-eosin staining and immunofluorescence staining for class III-β tubulin. The levels of inflammation-associated cytokines, such as interleukin (IL)-4, IL-6, TNF-α, IL-1β, IL-13 and IFN-γ were measured by flow cytometry. T cell proliferation was assessed by the carboxyfluorescein diacetate succinimidyl ester method. Finally, the functional role of EGCG in glaucoma was explored. The levels of the inflammation-associated proteins p-IκBα and p-p65 were measured by western blot analysis. The results showed that optic nerve injury occurred, and elevated levels of the inflammatory cytokines IL-4, IL-6, TNF-α, IL-1β, IL-13 and IFN-γ were observed in the rat model of acute glaucoma. In addition, an increased T lymphocyte proliferation rate and imbalance of Th1/Th2 cytokines were present in the models. Importantly, treatment with EGCG significantly alleviated optic nerve injury. At the molecular level, EGCG decreased the levels of inflammation-associated cytokines, decreased the proliferation rate of T lymphocyte cells, and repaired the imbalance of Th1/Th2 cytokines. Moreover, EGCG inhibited the increase in the phosphorylation of IκBα and p65 caused by modelling and thus suppressed the activation of the nuclear factor (NF)-κB signalling pathway. The findings of the present study indicate that EGCG could attenuate the symptoms of glaucoma and inhibit inflammatory responses by suppressing the NF-κB signalling pathway in a rat glaucoma model.

Introduction

Glaucoma is an irreversible ophthalmic disease leading to blindness, and there are several types of glaucoma, including congenital glaucoma, primary glaucoma, secondary glaucoma and mixed glaucoma. High intraocular pressure (IOP) is the primary cause of acute glaucoma and is accompanied by optic atrophy, degeneration and necrosis of retinal nerve layers (1-3). While decreasing the IOP is a key treatment for glaucoma, it is difficult to stop the progression of glaucoma (4). Several factors are implicated in the pathogenesis of glaucoma, such as impaired blood circulation, excitotoxic reactions caused by excessive accumulation of glutamate, free radical production, oxidative stress and immunological factors (5). Therefore, lowering the IOP is insufficient. Emerging evidence has indicated that proinflammatory factors play a key role in glaucoma development and progression. Identifying novel and effective therapeutic interventions to decrease inflammatory responses might be helpful for treating this disease and ultimately decrease the rate of blindness.

The nuclear factor κB (NF-κB) signalling pathway is critical for inflammation and is involved in various autoimmune diseases (6). The activation of NF-κB by different stimuli causes the phosphorylation and ubiquitination of IκB and subsequently activates the transcription and expression of several downstream genes. By this mechanism, the NF-κB signalling pathway directly regulates the expression of Th1- and Th2-type cytokines and the inflammatory response (7,8). In contrast, inhibition of the NF-κB signalling pathway by Pueraria and arylsulfonyl indoline-benzamide greatly suppresses the release of proinflammatory cytokines in mice with glaucoma and thus attenuates injury (1,9). Accordingly, the NF-κB signalling pathway plays an important role in glaucoma and may be a potential target for glaucoma therapy.

Epigallocatechin-3-gallate (EGCG), a major antioxidant catechin found in green tea (*Camellia sinensis*), has key anti-inflammatory (10,11), antioxidative (12,13),

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Abbreviations: IOP, intraocular pressure; NF-κB, nuclear factor κB; EGCG, epigallocatechin-3-gallate; CFSE, carboxyfluorescein diacetate succinimidyl ester

and anticancer (14-16) effects. It has been reported that EGCG attenuates hypothalamic inflammation by inhibiting the JAK2/STAT3 signalling pathways in microglia (17). Additionally, EGCG has been shown to exert beneficial effects against conditions such as liver inflammation (18), arthritis (19) and nephritis (20). It has been reported that EGCG can inhibit the phosphorylation and ubiquitination of IκB, thereby suppressing the activation of the NF-κB signalling pathway and decreasing the production of inflammatory cytokines (21). However, there have been few studies on whether EGCG can be used for glaucoma treatment.

The present study investigated the potential therapeutic effect of EGCG in glaucoma by using a rat glaucoma model established by high-pressure perfusion. Optic neuropathy was observed, and the levels of inflammatory-associated factors were measured in the glaucoma model rats treated with or without EGCG. Importantly, the mechanism by which EGCG relieves glaucoma was further explored. Overall, the present study provides a theoretical basis for glaucoma therapy.

Materials and methods

Establishment of a rat glaucoma model and drug treatment. Fifteen male Sprague-Dawley rats and 15 female Sprague-Dawley rats from Hunan SJA Laboratory Animal Co., Ltd. (6-8 weeks old; weight, 220-250 g) were used to establish the glaucoma model. The animals were housed in the standard animal facility with 12:12 h (light/dark cycle), 40-50% humidity, and 22°C temperature. The rats had free access to food and water. No obvious eye disease was observed before surgery. The rats were randomly divided into six groups (n=5 in each group): Control group, 1-day group, 3-day group, 7-day group, 14-day group, and 28-day group. The rats were anaesthetized via intraperitoneal injection of 2% pentobarbital sodium (50 mg/kg). Following anaesthesia, a needle was injected into the corneoscleral margin of the rat eyeball without touching the lens to prevent cataract formation. Normal saline was infused into the centre of the anterior chamber for 60 min. No iris or lens injury was induced during the operation (22). At the indicated time point (day 1, 3, 7, 14 or 28), all the rats in each group were anesthetized with 3% sodium pentobarbital (30 mg/kg) and 3-5 ml of abdominal aortic blood was collected. Then, eyeballs samples were collected after rats were sacrificed by cervical dislocation. The samples were used for assessment of pathological changes and inflammatory responses.

In order to evaluate the treatment effect of EGCG (Aladdin; cat. no. E107404-500 mg), 20 Sprague-Dawley rats (10 males and 10 females) were randomly divided into four groups (n=5 in each group): Control group (sham surgery without saline injection), glaucoma model group (no EGCG in drinking water), 14-day EGCG group (each rat was given 50 mg/kg/day EGCG by gavage for 14 continuous days immediately after modelling) (4), and 28-day EGCG group (each rat was given 50 mg/kg/day EGCG by gavage for 28 continuous days immediately after modelling). All animals in each group were sacrificed at the indicated time point (day 14 or 28), and samples were collected and used for assessment of pathological changes and inflammatory responses.

The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Please change it to The Third Xiangya Hospital of Central South University.

Haematoxylin-eosin (HE) staining. Rat eyeballs were collected from each group at specific time points and fixed in FAS fixation solution (Wuhan Servicebio Technology Co., Ltd.; cat. no. G1109) for >24 h at room temperature. PBS was used to rinse off the fixed solution and the tissues were embedded in paraffin. Embedded tissues were cut into 5-μm slices. The sections were stained with haematoxylin for 10 min at room temperature and rinsed by water, followed by eosin staining for 10 min at room temperature. The stained slices were washed once with 85% ethanol for 5 min at room temperature, and then washed once with 95% ethanol for 5 min at room temperature. Finally, the slices washed twice with 100% ethanol for 10 min each at room temperature, followed by imaging with light microscopy (BA400; McAudi Industrial Group Co., Ltd; magnification, x40).

Assessment of T cell proliferation by the carboxyfluorescein diacetate succinimidyl ester (CFSE) method. Rats in each group were sacrificed at specific time points, and the blood was collected from each rat to assess T lymphocyte proliferation. The procedure was performed as previously described (23). Briefly, cells were isolated by gradient centrifugation and counted with a cell counter. Cell suspension (1 ml) was added to the bottom of the tube. CFSE (cat. no. 423801) diluted with PBS was added (final CFSE concentration, 5 μmol/l), and the tube was rapidly vortexed to ensure homogeneous dispersal. After labelling with CFSE, the cells were treated with RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 5% foetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and cultured at 37°C in a 5% CO_2 incubator for 72 h. After that, the cells were incubated for 20 min at room temperature with an APC-conjugated anti-rat CD3 antibody (1 μg per million cells; cat. no. 201413; BioLegend, Inc.) and analysed within 1 h on a FACSCalibur flow cytometer (BD Biosciences). Acquired data were analyzed using FlowJo software (v7.6; FlowJo LLC).

Measurement of intracellular cytokine levels by flow cytometry. Rats in the control, 1-day, 3-day, 7-day, 14-day and 28-day groups were sacrificed on days 1, 3, 7, 14 and 28, respectively, and rats from the 14- and 28-day EGCG groups were sacrificed on days 14 and 28, respectively. Blood was collected from each rat and used to measure the levels of intracellular IL-4, IL-6, TNF-α, IL-1β, IL-13 and IFN-γ (custom panel; BioLegend, Inc.) and the levels of Th1- and Th2-type cytokines (cat. no. 740405; BioLegend, Inc.) were measured by flow cytometry. The protocol was performed as previously described (24,25).

Western blot analysis of inflammation-associated protein expression. The levels of the inflammation-associated proteins phosphorylated (p)-p65 (cat. no. 3033T), p65 (cat. no. 8242T), p-IκBα (cat. no. 9246S) and IκBα (cat. no. 4814T) (all from Cell Signaling Technology, Inc) were assessed by western blotting. The tissues were lysed in RIPA lysis buffer (Abcam) supplemented with protease inhibitor. The protein concentration of each sample was quantified by BCA assay with the BCA assay

kit (Bio-Rad Laboratories, Inc.). Equal amounts of proteins from each sample (20 mg) were loaded to 12% SDS-PAGE gels for electrophoresis and then transferred onto polyvinylidene difluoride (EMD Millipore) membranes. The membranes were blocked in 5% dry skimmed milk in TBST buffer (0.02 M Tris-base, pH 7.6, 0.8% NaCl and 0.1% Tween-20) for 1 h at room temperature. Specific primary antibodies (1:1,000) or a β-actin antibody (1:1,000; Cell Signaling Technology, Inc.) was added, and the membranes were incubated at 4°C overnight. After washing three times, the membranes were incubated with the appropriate HRP-conjugated secondary antibodies (1:3,000; cat. nos. BA1075 and BA1054; Boster Biological Technology) at room temperature for 1 h. Finally, the protein bands were detected by an enhanced chemiluminescence kit (cat. no. 32209; Thermo Fisher Scientific, Inc.) and then semi-quantified with ImageJ software (v1.8.0; National Institutes of Health).

Immunofluorescence staining. Rat eyeballs were fixed in FAS fixation solution for >24 h at room temperature, embedded in paraffin and sectioned into 5-μm slices. The sections were blocked in blocking buffer containing 5% BSA in PBS for 30 min at 37°C. The sections were then incubated with an anti-class III β-tubulin primary antibody (1:1,000; cat. no. ab18207; Abcam) overnight at 4°C (26). After washing three times, the sections were incubated with secondary antibody (1:2,000; cat. no. ab150077; Abcam) labelled with fluorescein at room temperature for 1 h in the dark. After washing, anti-fluorescence quenching agent (cat. no. G1401; Wuhan Servicebio Technology Co., Ltd.) was applied, and the sections were mounted on glass slides. Finally, the samples were imaged with a fluorescence microscope (magnification, x40; TE2000-U; Nikon Corporation).

Statistical analysis. For statistical analyses, one-way ANOVA with Tukey's post hoc test was performed using SPSS software (v12.0; SPSS Inc.). The results are presented as the means ± standard deviations of three independent experiments. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Successful establishment of an acute glaucoma rat model. The eyeballs of the animals were subjected to high-pressure perfusion to establish an acute glaucoma model (27). Rat eyeballs were removed for HE staining on days 1, 3, 7, 14 and 28 after modelling to observe the neuropathological changes. The results showed that gradual injury of the optic nerve, manifested as optic lesions and atrophy, occurred over time after modelling and that degeneration and necrosis of the retinal nerve layer also occurred (Fig. 1A). In addition, the fluorescence intensity of the class III-β tubulin protein in the 28-day group was lower compared with that in the control group (Fig. 1B), indicating that neuronal cells were damaged in the 28-day group. Flow cytometry revealed that the levels of intracellular cytokines, including IL-4, IL-6, TNF-α, IL-1β, IL-13 and IFN-γ, were gradually elevated following modelling, with the levels of IL-4 and TNF-α reaching the peak at day 14 and the rest peaking at day 28 (Fig. 1C). Furthermore,

the proliferation rate of T lymphocytes was measured by the CFSE method and found that the proliferation rate gradually increased with time after modelling (Fig. 1D). Total Th1 and Th2 cytokine levels were also assessed by flow cytometry. The results indicated that the levels of both Th1 and Th2 cytokines were increased during the early days after remodelling (Fig. 1E). However, the levels returned to baseline levels by the end of the experiment (Fig. 1E). The ratio of Th1 to Th2 cytokines was greatly diminished on day 1 and day 3 after remodelling but gradually recovered to a normal level and was increased by day 28, indicating an imbalance of Th1/Th2 cytokines developed during the progression of glaucoma (Fig. 1E). These results provide evidence that our animal model of acute glaucoma was successfully established.

EGCG suppressed the expression of inflammatory cytokines in rats with acute glaucoma. In order to study the role of EGCG in acute glaucoma, animals were fed with EGCG via gavage following modelling. The HE staining results showed that EGCG greatly attenuated the optical injury caused by high pressure, with better effects being observed following 28 days of treatment compared with after 14 days of treatment (Fig. 2A). In addition, the protein expression of class III-β tubulin was upregulated in the 28-day EGCG group compared with the model group, indicating that EGCG could repair the damage to the optic nerve induced by acute glaucoma (Fig. 2B). The cytokine levels were measured after EGCG treatment. The levels of cytokines, including IL-4, IL-6, TNF-α, IL-1β, IL-13 and IFN-γ, were significantly increased in the model group compared with the control group (Fig. 2C). However, these increases were significantly suppressed following treatment with EGCG for 14 days, and the levels of these cytokines returned to baseline levels after 28 days of EGCG treatment (Fig. 2C). The aforementioned results indicate that EGCG can suppress inflammatory responses in glaucoma and thus attenuate optical injury.

EGCG decreased T cell proliferation and the Th1/Th2 ratio in the peripheral blood of rats with glaucoma. The aforementioned results indicate that the proliferation rate of T lymphocytes was increased and that an imbalance of Th1/Th2 cells occurred in the glaucoma model group; it was then assessed whether EGCG regulated this process. As shown in Fig. 3A, treatment with EGCG for 14 days significantly decreased the T lymphocyte proliferation rate, and 28 days of treatment further decreased the rate (Fig. 3A). Furthermore, the increase in the levels of Th1-type and Th2-type cytokines following modelling were suppressed by EGCG treatment (Fig. 3B). The Th1/Th2 ratio was also restored by EGCG treatment (Fig. 3B). Altogether, these data demonstrate that EGCG can suppress T cell proliferation and restore the imbalance of Th1/Th2 cytokines during glaucoma.

EGCG inhibits the NF-κB signalling pathway. In order to study the mechanism by which EGCG suppressed inflammatory responses, the expression levels of inflammation-associated proteins were measured by western blot analysis. The results showed that p-IκBα/IκBα and p-p65/p65 levels gradually increased with time after the establishment of the glaucoma model (Fig. 4A). Notably, after treatment with EGCG, the
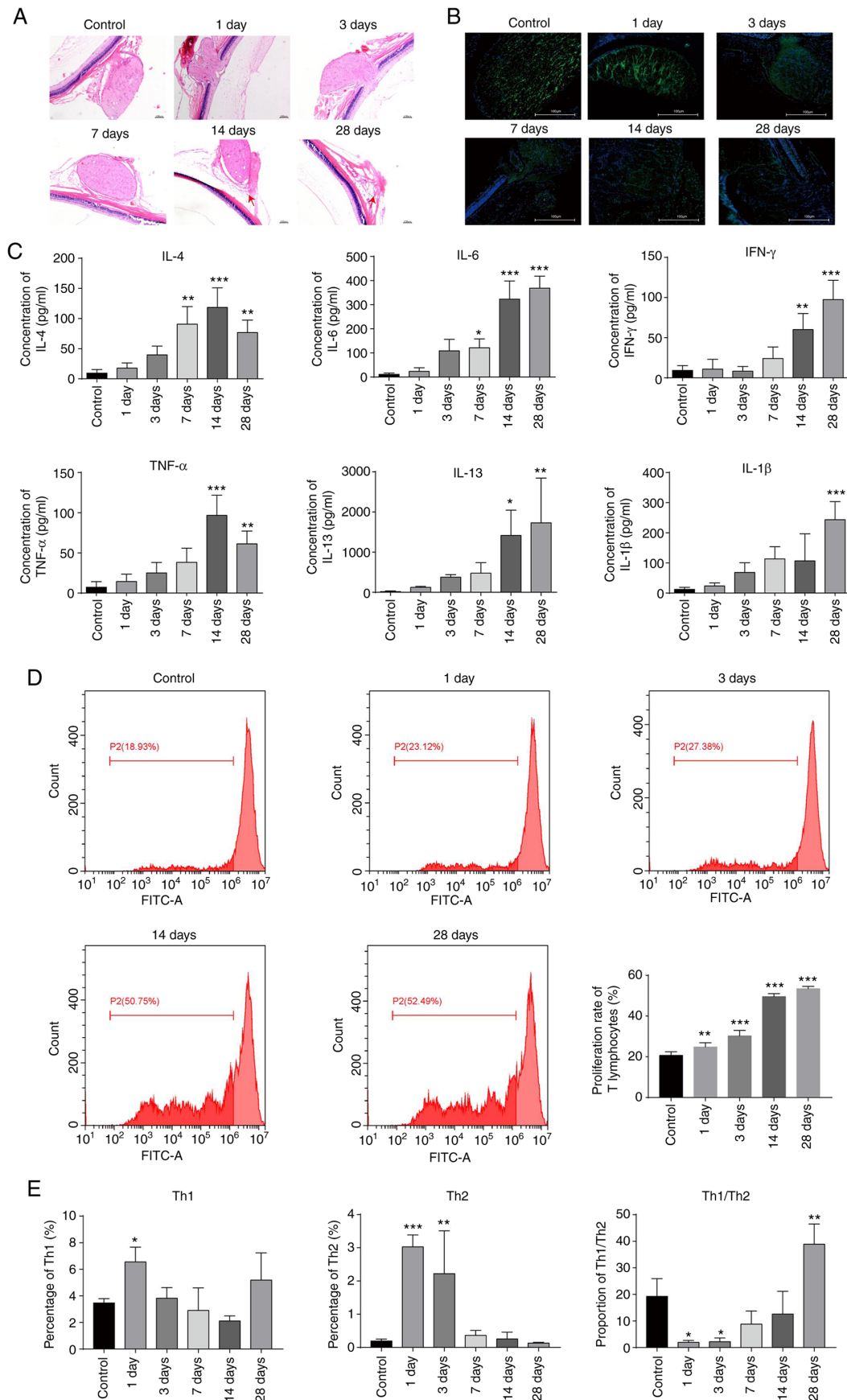
Figure 1. Successful establishment of an acute glaucoma rat model. (A) Observation of optic neuropathy by haematoxylin-eosin staining after modelling. Magnification, x40. (B) Measurement of class III β-tubulin levels by immunofluorescence staining after modelling. Magnification, x40 (C) Measurement of the levels of cytokines, including IL-4, IL-6, TNF-α, IL-1β, IL-13 and IFN-γ, by flow cytometry after modelling. (D) Assessment of T lymphocyte proliferation by the CFSE method on days 1, 3, 14, and 28 after modelling. The cells were treated with CFSE and analysed by flow cytometry. (E) The expression levels of Th1 and Th2 cytokines determined by flow cytometry on days 1, 3, 7, 14, and 28 after modelling. *P<0.05; **P<0.01; ***P<0.001. IL, interleukin; TNF, tumour necrosis factor; IFN, interferon; CFSE, carboxyfluorescein diacetate succinimidyl ester.
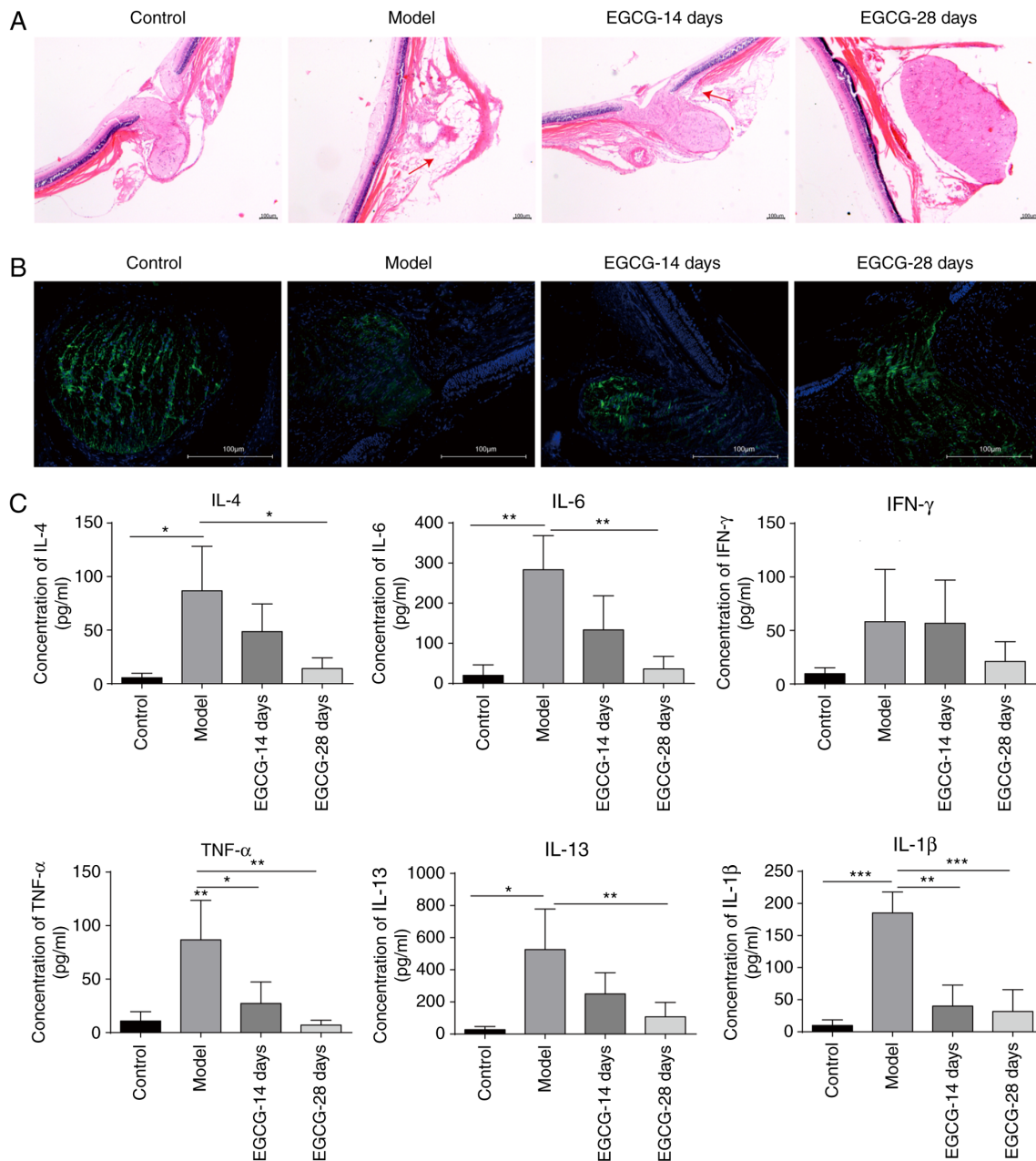
Figure 2. EGCG suppresses the expression of inflammatory cytokines in rats with acute glaucoma. (A) Observation of optic neuropathy by haematoxylin-eosin staining after treatment with EGCG. Magnification, x40. (B) Analysis of class III β-tubulin levels by immunofluorescence staining in the control group, model group and groups treated with EGCG for 14 and 28 days. Magnification, x40. (C) Measurement of the levels of cytokines, including IL-4, IL-6, TNF-α, IL-1β, IL-13 and IFN-γ, by flow cytometry after treatment with EGCG. *P<0.05; **P<0.01; ***P<0.001. EGCG, epigallocatechin-3-gallate; IL, interleukin; TNF, tumour necrosis factor; IFN, interferon.

increase in both p-IκBα/IκBα and p-p65/p65 levels induced by modelling were significantly inhibited (Fig. 4B). The afore-mentioned results indicated that the NF-κB signalling pathway is markedly activated in the course of glaucoma and that this activation can be inhibited by EGCG treatment.

Discussion

Glaucoma caused by high IOP is an optic nerve disease that can ultimately result in blindness. Currently, treatments for this disease include drugs (28) and surgery (29,30) to control IOP and limit optic nerve damage. However, prognosis remains to be improved, due to the various unwanted side effects of

these treatments (31). In the present study, through the use of an animal model, it was demonstrated that EGCG signifi-cantly ameliorates optic injury in the course of glaucoma by suppressing inflammatory responses and restoring the balance of Th1 and Th2 cytokines via the NF-κB pathway. The present study provides an avenue for the development of therapeutic strategies for glaucoma.

High IOP is a key risk factor for glaucoma. Thus, many studies have established animal models of glaucoma by increasing the IOP (32,33). It has been reported that IOP can be increased by injecting saline into the eyes. For example, Zhong (34) successfully generated an ocular hypertension rat model by injecting hypertonic saline into the episcleral
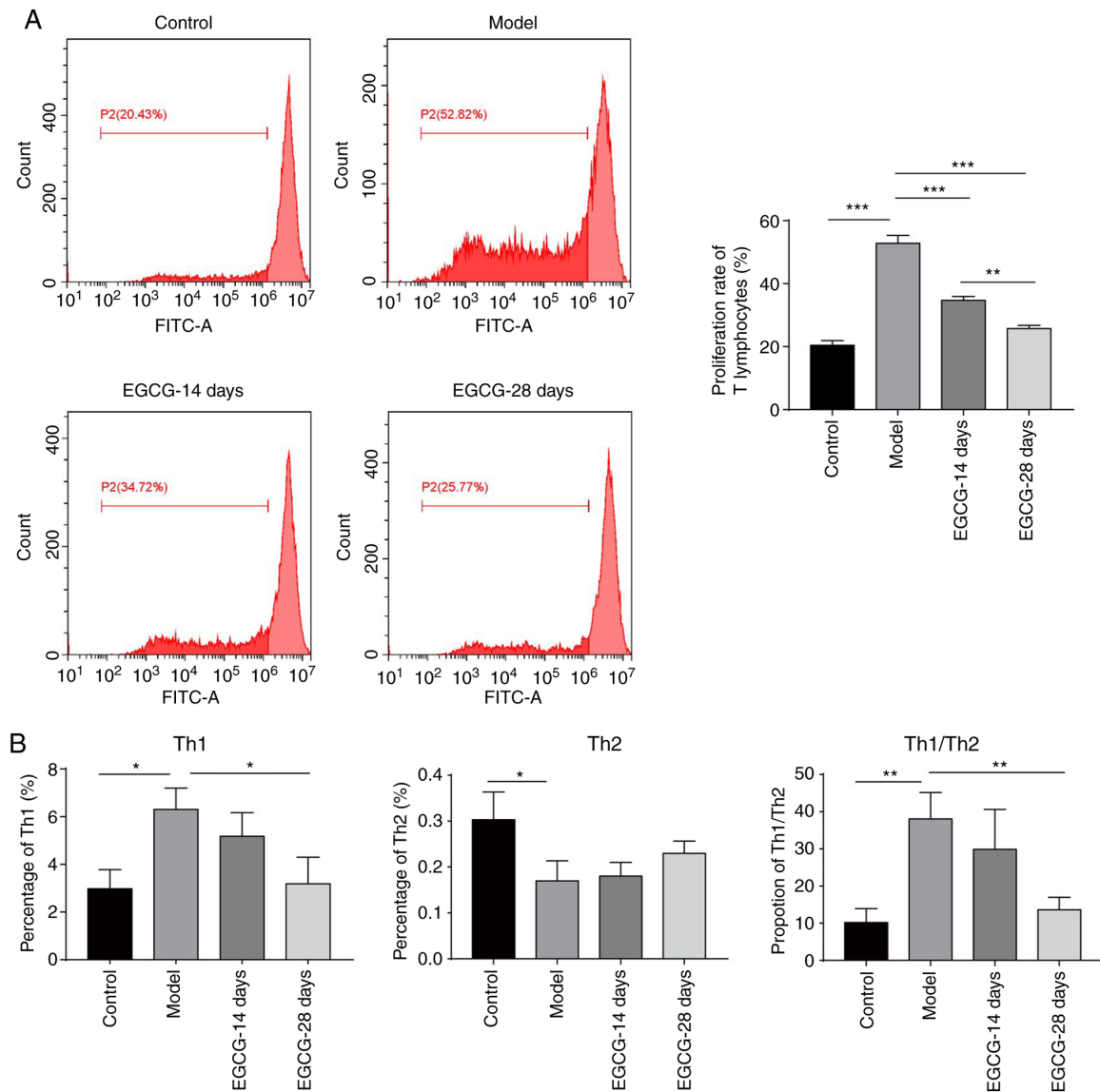
Figure 3. EGCG decreases T cell proliferation and the Th1/Th2 ratio in the peripheral blood of rats with glaucoma. (A) Analysis of T lymphocyte proliferation by the CFSE method in the control group, model group and groups treated with EGCG for 14 and 28 days. The cells were treated with CFSE and analysed by flow cytometry. (B) The expression levels of Th1 and Th2 cytokines determined by flow cytometry in the control group, model group and groups treated with EGCG for 14 and 28 days. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. CFSE, carboxyfluorescein diacetate succinimidyl ester; EGCG, epigallocatechin-3-gallate.

veins once weekly for two weeks. The success rate of this method was >80%, and IOP was elevated by 81% for 24 weeks. Similarly, Morrison *et al* (35) established an experimental rat model of acute glaucoma through injecting hypertonic saline into the aqueous outflow pathway, and the resulting inflammatory response led to increased IOP one week later. In the present study, acute glaucoma was modelled in rats by high-pressure infusion of saline. After infusion, optic nerve damage begins to develop, such as optic atrophy, degeneration and necrosis of the retinal nerve layer. In addition, a decrease in class III-β tubulin protein expression was observed, further indicating damage to optic nerves. Accompanying these pathological changes were marked increases in the levels of proinflammatory Th1/Th2 cytokines. These results indicate that the animal model was successfully established; thus, this approach for increasing IOP could be used as an alternative method for modelling acute glaucoma in animals.

EGCG is the main ingredient of green tea and has been shown to have a neuroprotective effect (36,37). For instance, Kian *et al* (38) found that EGCG may be effective in protecting neuronal cells against apoptosis and may increase neuronal survival time following nerve transection. Furthermore, Shen *et al* (4) observed that EGCG exerts a neuroprotective effect on retinal ganglion cells (RGCs) in chronic glaucoma. Consistently, the present study indicated that EGCG attenuates optic nerve injury induced by high IOP, as indicated by decreased optic atrophy, optic nerve degeneration and necrosis. Taken together, the present study results and those of previous studies indicate that EGCG has a conserved neuroprotective role. It might be interesting to examine whether EGCG can be applied for the treatment of other neurological diseases, such as Alzheimer's disease and Parkinson's disease.

Inflammation significantly contributes to the development and progression of glaucoma, and substantial changes
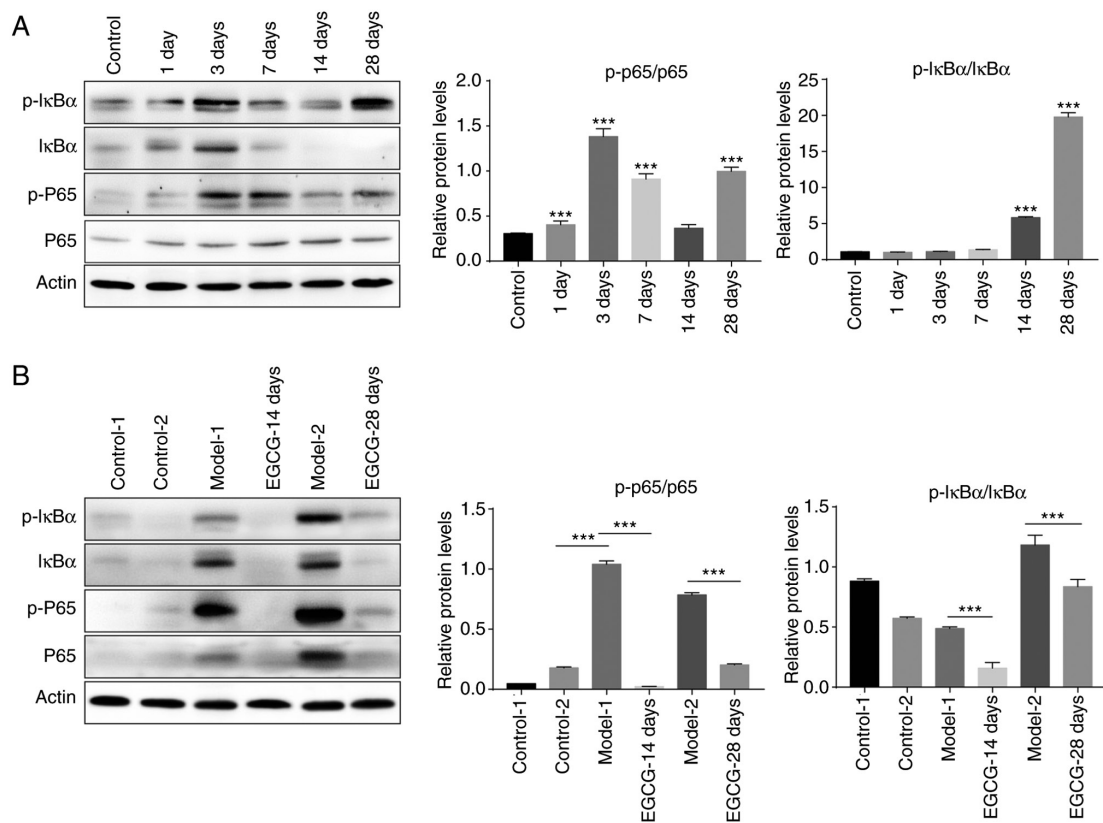
Figure 4. EGCG inhibits the nuclear factor-κB signalling pathway. (A) Expression levels of phosphorylated and unphosphorylated IκBα and p65 determined by western blot analysis on days 1, 3, 7, 14 and 28 after modelling. (B) Expression levels of phosphorylated and unphosphorylated IκBα and p65 determined by western blot analysis in the control group, model group, and groups treated with EGCG for 14 and 28 days. ***P<0.001 EGCG, epigallocatechin-3-gallate; p-, phosphorylated-.

in the levels of cytokines, such as IFN-γ, TNF-α, IL-1 and IL-6, have been reported (39,40). In patients with glaucoma, the expression of IL-4, IL-6, TNF-α, IL-1β, IL-13 and IFN-γ is upregulated, and this upregulation exacerbates the disease. The present study found that EGCG inhibits the increase in IL-4, IL-6, TNF-α, IL-1β, IL-13 and IFN-γ expression levels *in vivo*, suggesting that it suppresses inflammatory responses. In addition, other studies have shown that the T cell response plays a role in neurodegeneration (41,42). Indeed, T cell proliferation has been observed in glaucoma. T cell proliferation in the context of glaucoma could induce the production of proinflammatory cytokines and thus promote inflammation. The present study data showed that EGCG greatly decreases the proliferation rate of T cells. This might be one of the mechanisms by which the inflammatory responses are inhibited by EGCG in glaucoma. In addition, the Th1/Th2 ratio reflects inflammation *in vivo* and is associated with the progression of glaucoma. The finding that the Th1/Th2 ratio was restored by EGCG in the present glaucoma model further suggests that EGCG serves a neuroprotective role in glaucoma. This finding is consistent with growing evidence indicating that an imbalance in Th1/Th2 cytokine production contributes to glaucomatous optic neuropathy (43).

Activation of the NF-κB pathway plays a crucial role in inflammation, as it can promote the transcription and expression of several proinflammatory cytokines, which may further facilitate T-cell communication (44). The present study confirmed that the NF-κB pathway was activated in a rat model. Notably, EGCG treatment remarkably suppressed NF-κB pathway activation, indicating that EGCG inhibits inflammation by inhibiting the NF-κB signalling pathway. Future studies are required to determine whether other mechanisms are involved in the neuroprotective role of EGCG in glaucoma. Additionally, it remains to be further explored whether the neuroprotective effect of EGCG is dose-dependent.

In conclusion, the present study results demonstrate that EGCG attenuates optic nerve injury during glaucoma by suppressing the activation of the NF-κB signalling pathway and inflammatory responses. This study indicates that EGCG could serve as a promising adjuvant intervention for glaucoma treatment in the future.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

WHZ and YNC were responsible for the conception and design, and revision of the manuscript. WHZ prepared the draft of the manuscript and performed the major experiments that contributed to the acquisition, analysis and interpretation of data. YC and LMG assisted in the experiments and analyzed the data. WHZ and YNC confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), The Third Xiangya Hospital of Central South University [approval no. LLSC (LA) 2018-038].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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