Cxcl5/Cxcr2 Modulates Inflammation-Mediated Neural Repair After Optic Nerve Injury

Yu-Fen Liu
Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong
Jia-Jian Liang
Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong
Tsz Kin Ng
Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong
Zhanchi Hu
Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong
Ciyan Xu
Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong
Shaowan Chen
Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong
Shao-Lang Chen
Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong
Yanxuan Xu
Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong
Xi Zhuang
Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong
Shaofen Huang
Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong
Mingzhi Zhang
Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong
Chi Pui Pang
Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong
Ling-Ping Cen (✉ cenlp@hotmail.com)
Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong
https://orcid.org/0000-0003-3876-0606

Research

Keywords: Cxcl5, Cxcr2, retinal ganglion cells, microglia, optic nerve injury

Posted Date: December 16th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-126751/v1

Abstract

Background

Previous studies reported that mild inflammation promotes retinal ganglion cell (RGC) survival and axonal regeneration after optic nerve (ON) injury with involvement of infiltrating macrophages and neutrophils. Here we aimed to evaluate the involvement and regulation of the main inflammatory chemokine pathway Cxcl5/Cxcr2 in the inflammation-mediated RGC survival and axonal regeneration in mice after ON injury.

Methods

The expressions and cellular locations of Cxcl5 and Cxcr2 were confirmed in mouse retina. Treatment effects of recombinant Cxcl5 and Cxcr2 antagonist SB225002 were studied in the explant culture and the ON injury model with or without lens injury. The number of RGCs, regenerating axons, and inflammatory cells were determined, and the activation of Akt and Stat3 signaling pathways evaluated.

Results

Cxcr2 and Cxcl5 expressions were increased after ON and lens injury. Addition of recombinant Cxcl5 promoted RGC survival and neurite outgrowth in retinal explant culture with increase in the number of activated microglia, which was inhibited by SB225002 or clodronate liposomes. Recombinant Cxcl5 also alleviated RGC death and promoted axonal regeneration in mice after ON injury, and promoted the lens injury-induced RGC protection with increase in the number of activated microglia. SB225002 inhibited lens injury-induced cell infiltration and activation, and attenuated the promotion effect on RGC survival and axonal regeneration through reduction of lens injury-induced Akt activation.

Conclusions

Cxcl5 promotes RGC survival and axonal regeneration after ON injury and further enhances RGC protection induced by lens injury through microglia activation, which is attenuated by Cxcr2 antagonist. Cxcl5/Cxcr2 should be a potential therapeutic target for RGC survival promotion after ON injury.

Introduction

Optic nerve (ON) can be suffered from injury by multiple conditions, including trauma, ischemia, immune disorders, poisoning, tumor and metabolic disorders. Traumatic optic neuropathy has reported in 0.4 to 2.5% of facial trauma and 10% of craniofacial fractures [1, 2]. About 50% of individuals affected with traumatic optic neuropathy suffer permanent vision loss even after clinical treatments due to progressive degeneration of retinal ganglion cells (RGCs) and their axons [2]. As a part of the central nervous system, ON axons have poor regenerative ability after injury because of inhibition of axonal outgrowth by myelin proteins [3], scar formation [4] and lack of neurotrophic factors [5, 6]. Lens injury and zymosan injection have been reported to increase RGC survival and neurite outgrowth after ON injury, which could be

mediated through macrophage and neutrophil infiltration and activation [7–10]. Subsequent studies have identified oncomodulin combined with cyclic adenosine monophosphate (cAMP) elevation, ciliary neurotrophic factor (CNTF), chemokine SDF-1/CXCL12 contributing to this inflammation-mediated neural repair [11–14]. Yet, the mechanism of the inflammation-mediated neural repair still remain elusive.

Up-regulation of Cxcl5 has been reported in different inflammatory diseases, such as chronic obstructive pulmonary disease [15], inflammatory pain [16] and optic neuromyelitis [17]. Intraventricular injection of lipopolysaccharide, has also been demonstrated to increase Cxcl5 expression in the activated microglia [18]. In the neural system, recombinant Cxcl5 promoted the survival of rat cortical neuron in culture [19], and Cxcl5 secreted by adipose-derived stem cells promoted growth of axons in rat pelvic ganglion cells through the Jak/Stat pathway [20]. Since CXCR2, the receptor of CXCL5, was found to be expressed in human and rabbit retina [21], we hypothesized that Cxcl5 and Cxcr2 could be involved in the inflammation-mediated neural repair after ON injury. In this study, we investigated the effects of recombinant Cxcl5 and Cxcr2 antagonists on RGC survival and axonal regeneration *in vitro* and *vivo* after ON injury and in combination with the lens injury-induced intraocular inflammation.

Materials And Methods

Animals

Adult C57BL/6 mice (age: 8–12 weeks; average weight: 20–27 g) were purchased from Beijing Charles River Laboratory Animal Technology Co. Ltd., China. Mice were kept in standard condition with a 12-hr dark/light cycle at 21-23^{o}C, and fed food and water *ad libitum*. All animal experiments were conducted following the Statement on the Use of Animals in Ophthalmic and Vision research from Association for Research in Vision and Ophthalmology, and approved by the Animal Experimentation Ethics Committee of the Joint Shantou International Eye Center of Shantou University and the Chinese University of Hong Kong (approval no.: EC20140311(2)-P01). 4–6 mice were used for each group.

Gene expression analysis

The expression of Cxcr2 and Cxcl5 in the retinas of normal mice and the mice received optic nerve injury with or without lens injury was evaluated by SYBR green polymerase chain reaction (PCR). Briefly, total RNA of the retina was extracted by the Total RNA Isolation Nucleo Spin RNA II kit according to the manufacturer's instructions. The RNA was then converted to complementary DNA using the SuperScript III reverse transcriptase and subsequently amplified using SYBR Green I Master in the LightCycler 480 system with specific primers (**Table S1**), *Gapdh* was used as the house-keeping reference gene. Relative quantification of the gene expression was calculated using the ΔCt method.

Retinal explant culture

RGC survival was evaluated based on our previously established protocols [13, 22]. The retinas were dissected from intact mouse eyeballs with four cuts, and mounted onto nitrocellulose filter papers with

the RGC layer facing upwards. The retinas were cultured in Neurobasal-A media supplemented with B27, glutamine and 1x penicillin/streptomycin with or without the addition of recombinant Cxcl5 (1 μg/ml), Cxcr2 antagonist SB225002 (12.5 μM, 1.25 μM, 0.125 μM), clodronate liposomes or 0.1% DMSO (vehicle control) for 7 days at 37 °C in an incubator with 5% CO_2.

Neurite outgrowth was evaluated according to our previous study [22]. ON crush was performed 7 days before the collection of retina for the explant culture [23]. Seven days after ON crush, the retina was dissected with four cuts and mounted onto a nitrocellulose filter paper with RGCs facing upward. The mounted retina was then cut into eight equal pieces and divided into different treatment groups. The retina was placed at 5-mm distance on a culture plate coated with poly-lysine (200 μg/mL) and laminin (20 μg/mL) with the RGC layer facing downward. The culture condition was the same as RGC survival assessment.

After 7-day culture, the retinal explant was fixed with 4% paraformaldehyde (pH 7.4) for further staining by the same method on *in vivo* retina.

Surgeries

All surgeries were carried out under general anesthesia with the intraperitoneal injection of a mixture (1.5 ml/kg) of 100 mg/ml ketamine and 20 mg/ml xylazine. ON injury was performed according to our previous studies [8, 22]. Briefly, ON crush was performed with an angled jeweler's forceps (Dumont #5; Roboz, Rockville, MD) at 1 mm after the ON head for 5 seconds carefully without damaging the ophthalmic artery. The lens was injured by a 32 G needle bent at an angle of 90° and inserted behind the corneoscleral limbus of the eyeball. Lens injury was verified by direct observation through the cornea and confirmed by the occurrence of opacification within 1 week. For intravitreal injections, a 10-μl Hamilton syringe (Hamilton Company, Reno, NV) connected with a pulled glass pipette was inserted 1–2 mm behind corneaoscleral limbus aslant without infringing on the lens. One μl of recombinant Cxcl5 (2.5 μg/μl) was injected at 3 and 7 days after ON injury with the glass pipette changing each time. SB225002 (2 μg/g) [24–27] was injected intraperitoneally every day at 1 day before ON injury and for 14 days after ON injury. The mice were maintained for 14 days after ON injury before sacrificed for further experiments.

Retinal ganglion cell analysis

RGCs were evaluated by immunofluorescence analysis according to our previous studies [13, 22]. At 14 days after ON injury, the mice were perfused with saline and 4% paraformaldehyde, and the eyes were dissected for 2-hour post-fixation. The retinas were then dissected, blocked and permeabilized with 5% normal goat serum (NGS) and 0.2% Triton X-100 for 1 hour, incubated with βIII-tubulin antibody for overnight at 4 °C. After 3-time phosphate-buffered saline (PBS) washing, the retinas were incubated with the respective secondary antibody conjugated with Alexa Fluor Plus 555 for 2 hours. After PBS washing, the retinas were mounted on the slides with 50% glycerinum. Eight images (0.775×0.775 mm^2 each) were taken for each retina at 5 mm from the optic nerve head with 1 mm interval under a confocal

microscope (Leica TCS SP5 II). The number of RGCs in each image was counted manually with ImageJ software, and the average density of RGCs was determined.

Axonal regeneration analysis

ON was dissected from the eyeball, fixed in 4% paraformaldehyde in PBS overnight, cryoprotected with the 10–30% sucrose gradient and embedded in optimal cutting temperature compound. Five sections (10 μm) per ON were obtained longitudinally. The ON sections were stained according to our previous studies [8, 28]. Briefly, the cryo-sections were blocked in 10% donkey serum for 1 hour and incubated with GAP-43 antibody in 5% donkey serum for overnight at 4 °C, followed by 2-hour incubation of secondary antibody conjugated with Alexa Fluor Plus 555 at room temperature. The stained ON sections were imaged by a confocal microscope (Leica TCS SP5 II). The regenerating axons were counted at the distal sites of 0.1, 0.2, 0.5 and 1 mm from the crushed site. Total number of regenerating axons at each site were calculated with the formula below. The width at every site was measured that r is the radius of the nerve, and t is the section thickness.

$\Sigma ad = \pi r^2(\text{average axons/mm width})/t$

Retinal section immunofluorescence analysis

To determine the retinal localization of Cxcr2 and Cxcl5, immunofluorescence analysis on the paraffin sections was performed. Briefly, the eyeballs were fixed in 10% formaldehyde for more than 24 hours, followed by the dehydration in the ethanol gradient (50%, 75%, 85%, 95%, 100%, 100%) and in xylene. After embedded in paraffin, the eyeballs were sectioned with 4 μm thickness by a microtome (LeicaRM2235). After dewaxed by xylene, the eyeball sections in pupil-optic nerve position were treated with heat-induced epitope retrieval followed with immunofluorescent analysis method mentioned above. One image cross the optic nerve and two in peripheral were taken under a confocal microscope (Leica TCS SP5 II).

To study the inflammatory response in different groups, the time courses of inflammatory cell infiltration was explored first. We found that the inflammatory cell infiltrating largely at Day 5–9 (**Figure S1**), so Day 7 was chosen to study the inflammatory response under different conditions. The retina frozen sections were harvested 7 days after ON injury then stained with rat anti-CD68 antibody as mentioned above.

Immunoblotting analysis

At 7 days after ON injury, the retina was dissected and homogenized in cold radio immunoprecipitation (RIPA) lysis buffer supplemented with protease and phosphatase inhibitors. Total protein concentrations were evaluated by the Micro BCA Protein Assay Kit. After denaturating at 99 °C for 5 minutes, equal amount of total proteins (20 *μg*) in each samples were resolved using 8% SDS-PAGE and blotted onto the nitrocellulose membranes. The membranes were blocked in 5% non-fat milk solution and incubated with primary antibodies of Stat3, phospho-Stat3, Akt, phospho-Akt, followed by respective horseradish peroxidase-conjugated secondary antibodies. The signal was visualized by enhanced

chemiluminescence in ChemiDoc™ XRS + system (Bio-Rad). The densitometry was determined and normalized to Gapdh expression.

Cell viability analysis

To confirm the specificity of SB225002, the viability of cells with (HaCaT) or without (B3) Cxcr2 expression after SB225002 treatment was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay. Briefly, 30,000 cells in each well were resuspended in the 24-well plate with completed medium for 24 hours at 37 °C in an incubator with 5% CO_2, and then treated with SB225002 (12.5 μM) for another 24 hours. After 24-hour treatment, MTT solution was added to each well and cultured for 3 hours at 37 °C, and dissolved in 0.5 ml isopropanol. 100 μl solution per well was transferred to the 96-well plate and the intensity was measured by absorbance at 570 nm with a reference of 630 nm.

Statistical analysis

Data was presented as mean ± standard deviation (SD). After verifying the data normality and homogeneity of variance, one-way analysis of variance (ANOVA) with post-hoc Bonferroni test or nonparametric Kruskal-Wallis test with Dunn's multiple-comparisons test were applied to compare results of different groups. Independent T-test was used for the comparison between two groups. All statistical analyses were performed using a commercially available software (IBM SPSS Statistics 23; SPSS Inc., Chicago, IL). $P < 0.05$ was considered as statistical significance.

Results

Expression of Cxcl5 **and** Cxcr2 **in mouse retina**

Expressions of both *Cxcl5* and *Cxcr2* genes were detected in normal mouse retina, but their expressions showed no significant difference after ON injury (*Cxcl5*: $P = 0.111$; *Cxcr2*: $P = 0.188$) as compared to those without ON injury; however, with lens injury, the expression of both *Cxcl5* ($P = 0.004$) and *Cxcr2* ($P = 0.001$) increased significantly as compared to those without ON and lens injury (Fig. 1A and **B**). Immunofluorescence analysis revealed that Cxcl5 expressed in the GC layer (GCL), inner nuclear layer (INL), outer plexiform layer (OPL) and photoreceptor layer (PRL; Fig. 1C-H), whereas Cxcr2 expressed in GCL, some cells in the IPL, INL and outer nuclear layer (ONL, Fig. 1I-N). In addition, the infiltrating cells also expressed Cxcl5 and Cxcr2 in the retina of the mice with ON and lens injury, and the expression of Cxcr2 was co-localized with the markers for infiltrating macrophages and neutrophils (**Figure S2**).

Cxcl5 promoted ex vivo **retina ganglion cell survival and neurite outgrowth**

After 7-day treatment of recombinant Cxcl5, the number of RGCs ($P < 0.001$) was significantly higher than that of control group by 37.9%. In addition, the number of neurite outgrowth in Cxcl5-treated group was 2.80 times higher than that in the control group ($P = 0.017$), and the average axon length was 1.73 times longer than that in the control group ($P = 0.01$). Meanwhile, immunofluorescence analysis on CD68

showed that the number of activated retinal microglia cells in the GCL increased by 40.3% as compared to the control group ($P = 0.034$; Fig. 2A-H, Q **and R**). Our results showed that Cxcl5 could promote RGC survival and neurite regeneration in retinal explant culture.

Cxcr2 antagonist attenuated ex vivo **RGC survival and microglia activation**

To explore whether Cxcl5 protects RGCs through its receptor Cxcr2, the effect of Cxcr2 antagonist SB225002 on RGC survival was evaluated in the retinal explant culture. Immunofluorescence analysis showed that SB225002 caused a concentration-dependent inhibitory effect on RGC survival. There was no significant difference in the number of RGCs between 0.125 μM SB225002 and the vehicle groups ($P > 0.999$). Instead, the number of RGCs in the 1.25 and 12.5 μM groups decreased by 26.3% ($P < 0.001$) and 32.9% ($P < 0.001$) respectively, as compared to the vehicle group. Meanwhile, the number of activated microglia also significantly decreased by 73.0% in the 1.25 μM ($P < 0.001$) and 83.0% in the 12.5 μM ($P < 0.001$) groups, as compared to the vehicle group. There were no statistically significant differences in the number of RGCs ($P > 0.999$) and microglia ($P = 0.817$) between the vehicle group and the control group (Fig. 2I-R**)**. Our results indicated involvement of Cxcr2 in the regulation of RGC survival in the retinal explant culture.

To further confirm the cell survival regulatory effect of SB225002 specifically through Cxcr2, we determined *Cxcr2* gene expressions in 11 cell lines. We identified HaCaT as *Cxcr2* expressing cells and B3 as cells not expressing *Cxcr2*. MTT assay showed that viability of HaCaT cells treated with 12.5 μM SB225002 was significantly lower than that of control group ($P = 0.005$); in contrast, there was no significant difference in B3 cell viability among different SB225002 concentrations ($P = 0.41$; **Figure S3**), suggesting that SB225002 reduces cell viability specifically through Cxcr2.

Cxcr2 antagonist and clodronate liposomes attenuated the promotion effect of Cxcl5 in retinal explant culture

To confirm the RGC protective effect of Cxcl5 acting through Cxcr2, the effect of co-treatment of recombinant Cxcl5 and SB225002 was assessed. The number of RGCs in the co-treatment of recombinant Cxcl5 and 12.5 μM SB225002 was 63.4% lesser than that in the Cxcl5 alone group ($P < 0.001$) and 52.3% lesser than the control group ($P < 0.001$). Similarly, the number of $CD68^+$ microglia in the co-treatment of recombinant Cxcl5 and 12.5 μM SB225002 was 72.3% lesser than that in treated with Cxcl5 alone ($P < 0.001$) and 61.2% lesser than the control group ($P = 0.001$; Fig. 2C, G, Q **and R**).

To delineate whether microglia participate in the Cxcl5-mediated RGC protection, clodronate liposome was applied to remove microglia. The number of $CD68^+$ microglia in the co-treatment of recombinant Cxcl5 and clodronate were 72.3% lesser than that in the Cxcl5 alone group ($P < 0.001$), and 61.2% lesser than the control group ($P < 0.001$). Meanwhile, the number of RGCs in the co-treatment of recombinant Cxcl5 and clodronate was 63.4% lesser than that in the Cxcl5 group ($P < 0.001$), and 52.3% lesser than the control group ($P < 0.001$; Fig. 2D, H, Q **and R**). As the majority of microglia had been eliminated by

clodronate liposomes, the promoting effect of Cxcl5 on RGC survival ceased. Therefore, our results indicated that Cxcl5 promotes RGC survival may through the activation of microglia.

Cxcl5 promoted retina ganglion cell survival and axonal regeneration after optic nerve and lens injury

We evaluated the effect of Cxcl5 on RGC survival and axonal regeneration in mice with optic nerve and lens injury. At 3 and 7 days after ON injury or ON and lens injury, recombinant Cxcl5 protein was injected intravitreally, and the retinas harvested 14 days after ON injury. The number of RGCs in ON-injured mice treated with Cxcl5 was increased by 23.1% as compared to those treated with vehicle ($P = 0.02$; Fig. 3A, B **and I**). Remarkably, the number of RGCs in the ON and lens-injured mice treated with Cxcl5 increased by 21.4% as compared to those treated with vehicle ($P = 0.001$; Fig. 3C, D **and I**). For the inflammatory response, immunofluorescence analysis on CD68 showed that the number of activated microglia cells in ON-injured mice treated with Cxcl5 was 63 times higher than those with ON injury alone ($P = 0.002$); however, there was no significant difference on the number of infiltrating $CD68^+$ cells in the vitreous cavity between these two groups ($P = 0.924$). On the contrary, the number of microglia cell activation in ON crush and lens-injured mice treated with Cxcl5 was 24 times higher than those with ON and lens injury alone ($P = 0.004$), and the number of infiltrating $CD68^+$ cells in the vitreous cavity of the ON crush and lens-injured mice treated with Cxcl5 was 52% higher than those with ON and lens injury alone ($P = 0.014$, ; Fig. 3E-H, J **and K**). These suggested that Cxcl5 could promote RGC survival in mice after ON injury largely may due to microglia activation and partially through the infiltrating inflammatory cells induced by lens injury.

For axonal regeneration, the number of $GAP43^+$ regenerating axons at 0.1 mm from the crushed site in ON-injured mice treated with Cxcl5 was significantly higher than those with ON injury alone ($P = 0.025$), although there were no significant differences at other distances ($P > 0.05$; Fig. 4A, B **and G**). Besides, Cxcl5 did not further promote axonal regeneration in ON-injured mice with lens injury, indicating that Cxcl5 did not enhance the inflammation-mediated axonal regeneration in RGCs after ON injury (Fig. 4C, D **and G**).

Cxcr2 antagonist SB225002 inhibit the promotion effect of Cxcl5 in vivo

In normal mice received intraperitoneal injection of 2 μg/g SB225002, there was no statistical difference in the number of RGCs group as compared to the normal mice without SB225002 treatment ($P = 0.948$; Fig. 5A, B **and H**), suggesting that 2 μg/g SB2250022 did not affect RGC survival in normal mice. Therefore, 2 μg/g SB225002 was chosen for further studies.

Immunofluorescence analysis on βIII-Tubulin revealed that SB225002 treatment showed no significant difference on the number of RGCs after ON injury as compared to the mice with ON injury alone ($P = 0.871$). Instead, SB225002 significantly attenuated the effect of lens injury on RGCs that the number of RGCs in ON and lens-injured mice treated with SB225002 was 38.7% lesser than that those treated with vehicle ($P = 0.0009$; Fig. 5C-H). Moreover, the number of regenerating axons in ON and lens-injured mice

treated with SB225002 at 0.1 mm (P = 0.006), 0.2 mm (P = 0.012), 0.5 mm (P = 0.049) and 1 mm (P = 0.006) was significantly reduced as compared to the mice with ON and lens injury only (Fig. 4C, E, F, G). In addition, Immunofluorescence analysis on CD68 showed that macrophage infiltration was significantly inhibited by SB225002 in ON and lens-injured mice as compared to those treated with vehicle (P = 0.001, Fig. 6A-I, All cell counting results are available in supplementary tables). Collectively, these suggested that SB225002 would inhibit the promotion effect of lens injury on RGC survival and axonal regeneration.

Cxcr2 antagonist reduced the lens injury-induced Akt activation

Akt activation (phospho-Akt/total Akt) in mice with ON and lens injury was 1.99 folds higher than those with ON injury only (P = 0.026). With 7 day SB225002 treatment, the increased Akt activation was significantly reduced as compared to those of ON and lens-injured mice (P = 0.048). However, there was no statistically significant difference in Stat3 activation (phospho-Stat3/Stat3) among different treatment groups (P = 0.054, Fig. 7). These results suggested participation of Akt activation in the protection of ON injury by Cxcl5/Cxcr2.

Discussion

Results from this study demonstrated that: 1) Cxcl5 and its receptor Cxcr2 are expressed in the retinas of normal C57BL/6 mice, and their expression significantly increase after lens injury. 2) Cxcl5 promotes *ex vivo* RGC survival, neurite outgrowth and microglia activation, which are attenuated by Cxcr2 antagonist SB225002 and clodronate liposome. 3) Intravitreal injection of recombinant Cxcl5 reduces RGC death and promotes axonal regeneration with the activation of microglia after ON injury, and enhances the RGC protective effect by lens injury. 4) Intraperitoneal injection of Cxcr2 antagonist SB225002 attenuates the RGC protective effect by lens injury by inhibiting inflammatory cell infiltration and microglia activation. 5) Intraperitoneal injection of SB225002 reduces the increased Akt activation induced by lens injury. Collectively, Cxcl5/Cxcr2 could be involved in the inflammation-mediated RGC survival and axonal regeneration after ON injury.

Normal C57BL/6 mice express *Cxcl5* and *Cxcr2*, and the expression increased after lens injury (Fig. 1A, B). Our results are similar to other studies in that Cxcl5 expression increased in lung after pneumonia induction [29] and in peritoneal exudates after methylated bovine serum albumin injection in mice [30]. We confirmed location of Cxcl5 in the GCL, IPL and OPL as well as the infiltrating cells. Similar to a reported study in rabbit [21], Cxcr2 was expressed in GCL, individual cells in IPL, INL and ONL (Fig. 1C-N). We also found its expression in both macrophages and neutrophils (**Figure S2**).

In the neural system, recombinant Cxcl5 promoted the survival of rat cortical neuron pelvic ganglion cells in culture [19, 20]. In this study, we further demonstrated the protective effect of Cxcl5 upon ON injury *in vitro* and *in vivo*, confirming the neuronal protective effect of Cxcl5. Moreover, we also observed increase in activated microglia in the retina after recombinant Cxcl5 treatment both *in vitro* (Fig. 2) and *in vivo*

(Fig. 3). Cxcl5 not only attracts infiltrating inflammatory cells in lung as reported [29], but also activates the resident microglia in the retina. As Cxcl5 expression in brain microglia increased after LPS induction [31], there could be a positive feedback response on Cxcl5 expression in microglia during inflammation.

SB225002, a potent selective Cxcr2 antagonist, inhibited Cxcr2 function by competitive binding of Cxcr2 with the Cxcr2 ligands [24, 25]. In this study, enhanced RGC survival and microglia activation by Cxcl5 treatment were attenuated by SB225002 administration in explant culture (Fig. 2), indicating involvement of resident microglia in the retina in protection of RGC against Cxcl5. Such postulation was further confirmed by the removal of microglia with the addition of clodronate liposome (Fig. 2). The association of microglia activation with the increased RGC survival by Cxcl5 was also found in the ON injury mouse model. When lens injury induced the infiltration of macrophage and promoted RGC survival, Cxcl5 could further enhance the lens injury-induced effects (Fig. 3). Therefore, the infiltrating macrophage and resident microglia could both participate in the inflammation-mediated protection for RGCs. As Cxcr2 antagonist attenuated the increased RGC survival, axonal regeneration and inflammatory responses by Cxcl5 and LI (Fig. 4–6), Cxcl5 modulated lens injury-induced RGC protection through Cxcr2. Nevertheless, whether other Cxcr2 ligands would also participate in the inflammation-mediated RGC protection requires further investigations.

Previous studies suggested that microglia play a causative role in central nervous system diseases. Intraperitoneal injection of LPS combined with cerebral ischemia-hypoxia injury in rat pups led to increased Cxcl5 expression and microglia activation in white matter, accompanying with the damage of blood-brain barrier [31]. Severe nerve injury accompanied with necrotizing enterocolitis might be related to microglia activation in the brain induced by TLR4 endogenous ligand produced after intestinal injury [32]. However, microglia could be involved in the maintenance of the homeostasis in the central nervous system and play a protective role by promoting nerve growth, removing tissue debris and regulating inflammatory response under various pathological conditions [33]. Three-dimensional electron microscopy has visualized the replacement of presynaptic terminal of cortical neurons by activated microglia in adult mice, triggering the gamma spectrum of cortical neurons to discharge, which in turn increased the activity of neurons and promoted secretion of anti-apoptotic and nerve nutrition elements [34]. Inflammation could be beneficial or damaging to tissue or even organs under different pathophysiological conditions [35]. There are different subtypes of microglia, such as M1 pro-inflammation type and M2 anti-inflammation type [36]. Further studies are needed to evaluate the proportions of different subtype microglia under different treatments so as to delineate the mechanism of microglia on RGC protection.

Conclusion

This study, for the first time, revealed that recombinant Cxcl5 promotes RGC survival and axonal regeneration after ON injury through microglia activation *in vitro* and *in vivo*, which could be eliminated by Cxcr2 antagonist SB225002 and clodronate liposomes. Recombinant Cxcl5 could also enhance the RGC

protective effect by lens injury, and the lens injury-induced Akt activation would be inhibited by Cxcr2 antagonist. Our results suggest Cxcl5/Cxcr2 to be potential therapeutic targets for ON injury.

Abbreviations

RGC, retinal ganglion cell; Cxcl5, chemokine ligand 5; Cxcr2, chemokine receptor 2; ONC, optic nerve crush; LPS, lipopolysaccharide; ON, optic nerve; ONC, optic nerve crush; LI, lens injury; cAMP, cyclic adenosine monophosphate; CNTF, ciliary neurotrophic factor; RIPA, radioimmunoprecipitation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Declarations

Acknowledgements

This study was supported by the National Natural Science Foundation of China (81570849), Natural Science Foundation of Guangdong Province (2015A030313446, 2020A1515011413), Joint Regional Basic Science and Applied Basic Science Research Fund of Guangdong Province (2019A1515110685), Special Fund for Chinese Medicine Development of Guangdong Province (20202089), and Grant for Key Disciplinary Project of Clinical Medicine under the Guangdong High-level University Development Program, China.

Authors' contributions

YFL, JJL, TKN and LPC conceived and designed experiments and analyzed data. YFL, JJL, ZH, CX, SC, SLC, YX, XZ and SH performed experiments. YFL, TKN, CPP and LPC drafted the manuscript. The authors read and approved the final manuscript.

Funding

This study was supported by the National Natural Science Foundation of China (81570849), Natural Science Foundation of Guangdong Province (2015A030313446, 2020A1515011413), Joint Regional Basic Science and Applied Basic Science Research Fund of Guangdong Province (2019A1515110685), Special Fund for Chinese Medicine Development of Guangdong Province (20202089), and Grant for Key Disciplinary Project of Clinical Medicine under the Guangdong High-level University Development Program, China.

Availability of data and materials

The datasets used and/or analyzed during the current study are available through the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong, Shantou, Guangdong, China.[2]Shantou University Medical College, Shantou, Guangdong, China.[3]Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong.

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Figures

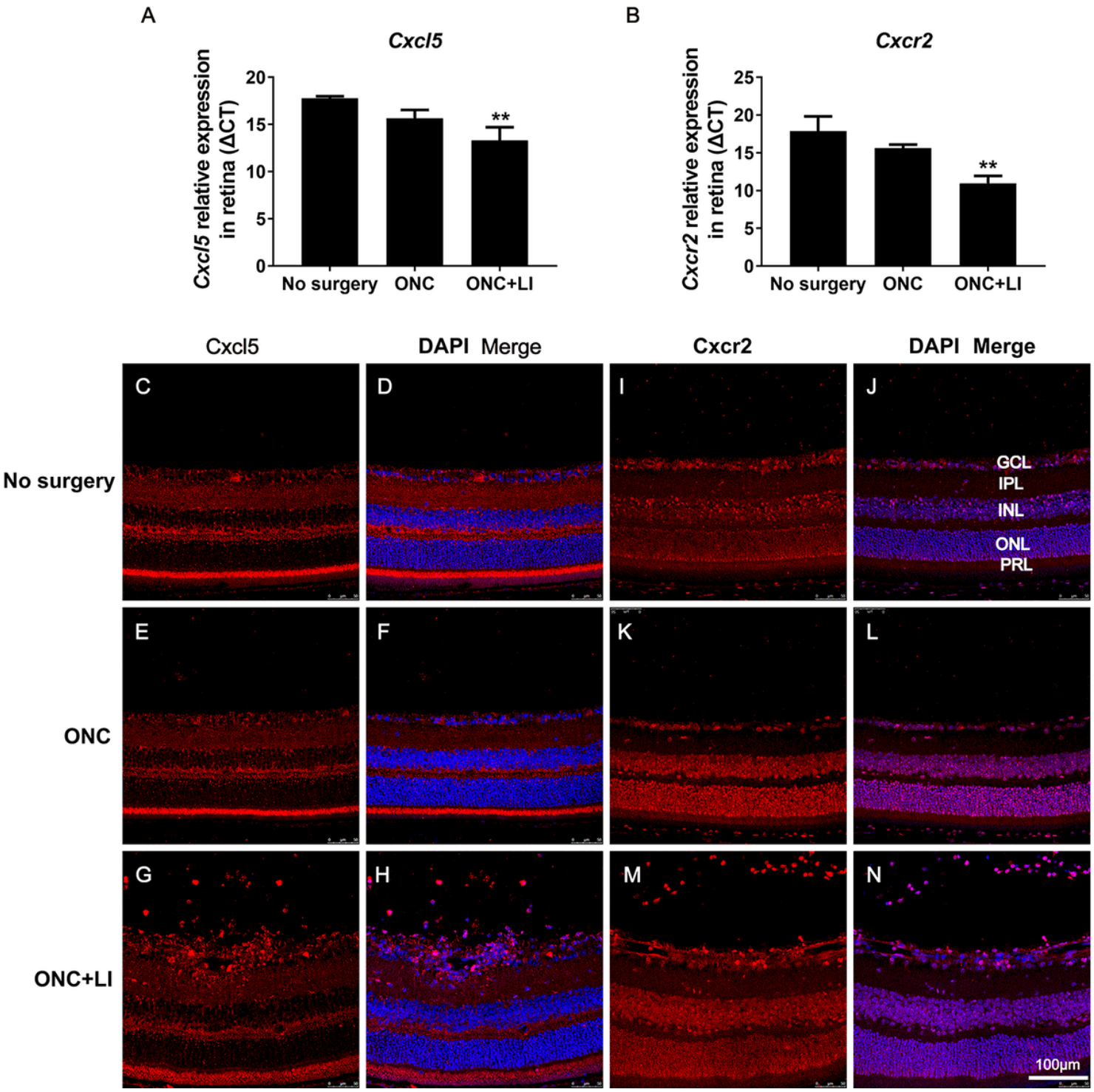
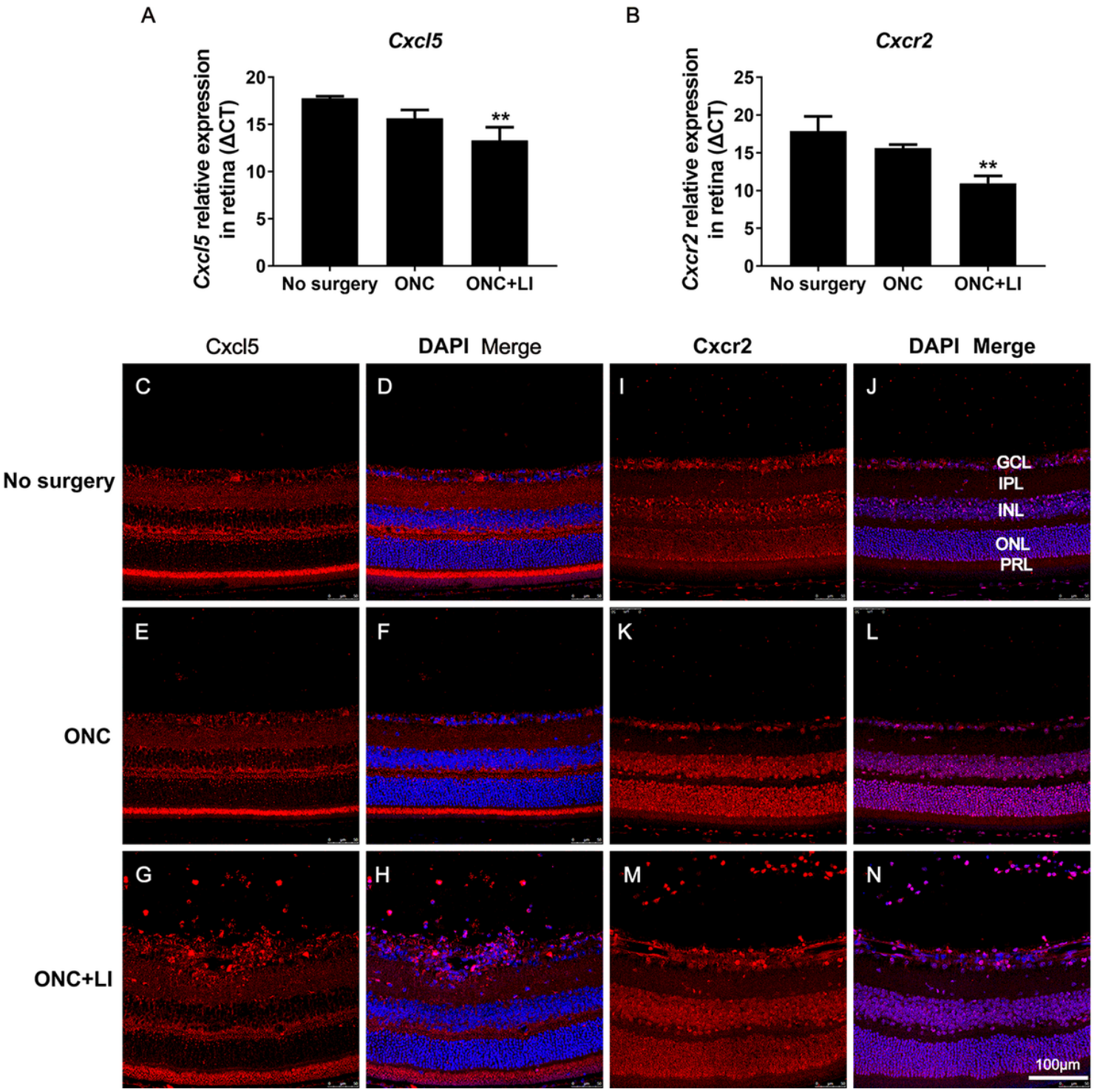


Figure 1

Cxcl5 and Cxcr2 expression in the retina. (A and B) SYBR green PCR showed that normal mice retina expressed Cxcl5 and Cxcr2. The expression increased mildly after ONC, and significantly increased after LI. **: P < 0.01, compared with no surgery group. (C-N) Immunofluorescent staining showed that Cxcl5 expressed in GCL, INL, OPL, PRL and (I-N) Cxcr2 expressed in GCL, Individual cells on the IPL, INL and ONL. (G, H, M, N) Cxcl5 and Cxcr2 also expressed in the infiltrating cells in LI groups. GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL□outer plexiform layer, PRL: photoreceptor layer. Scale bar: 100 μm.



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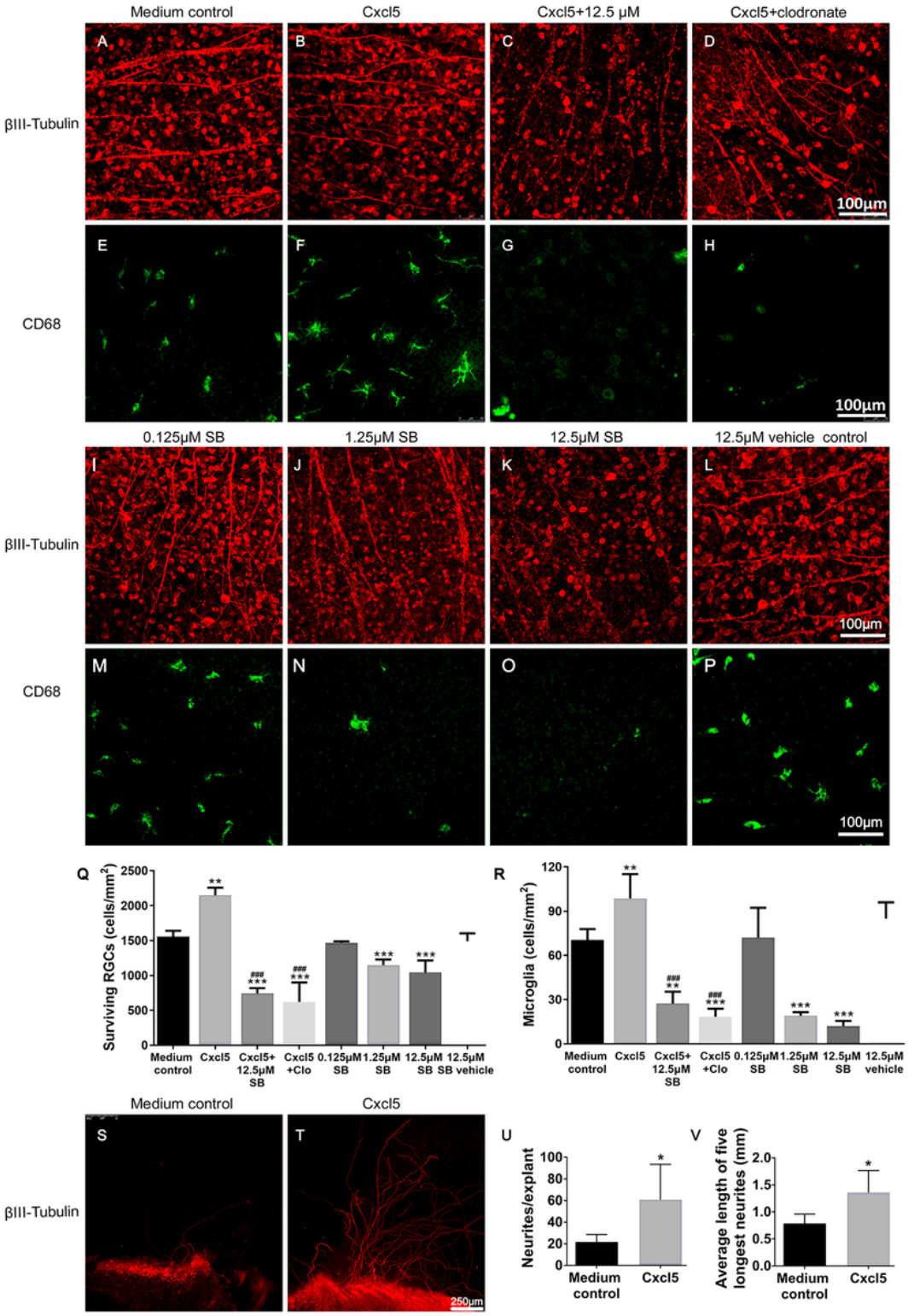

Figure 2

RGCs survival, axonal regeneration and microglia activation in retinal explant culture with different treatments. (A-D, I-L and Q) Immunofluorescent analysis showed βIII tubulin+ RGCs in retina explants in medium control, Cxcl5, Cxcl5+SB225002, Cxcl5+Clodronate groups and different concentration Cxcr2 antagonist SB225002. Scale bar: 100 μm, **: P < 0.00, ***: P < 0.001, compare with medium control group, ###: P < 0.001, compare with Cxcl5 group. (E-H, M-P and R)CD68+ activating microglia in medium

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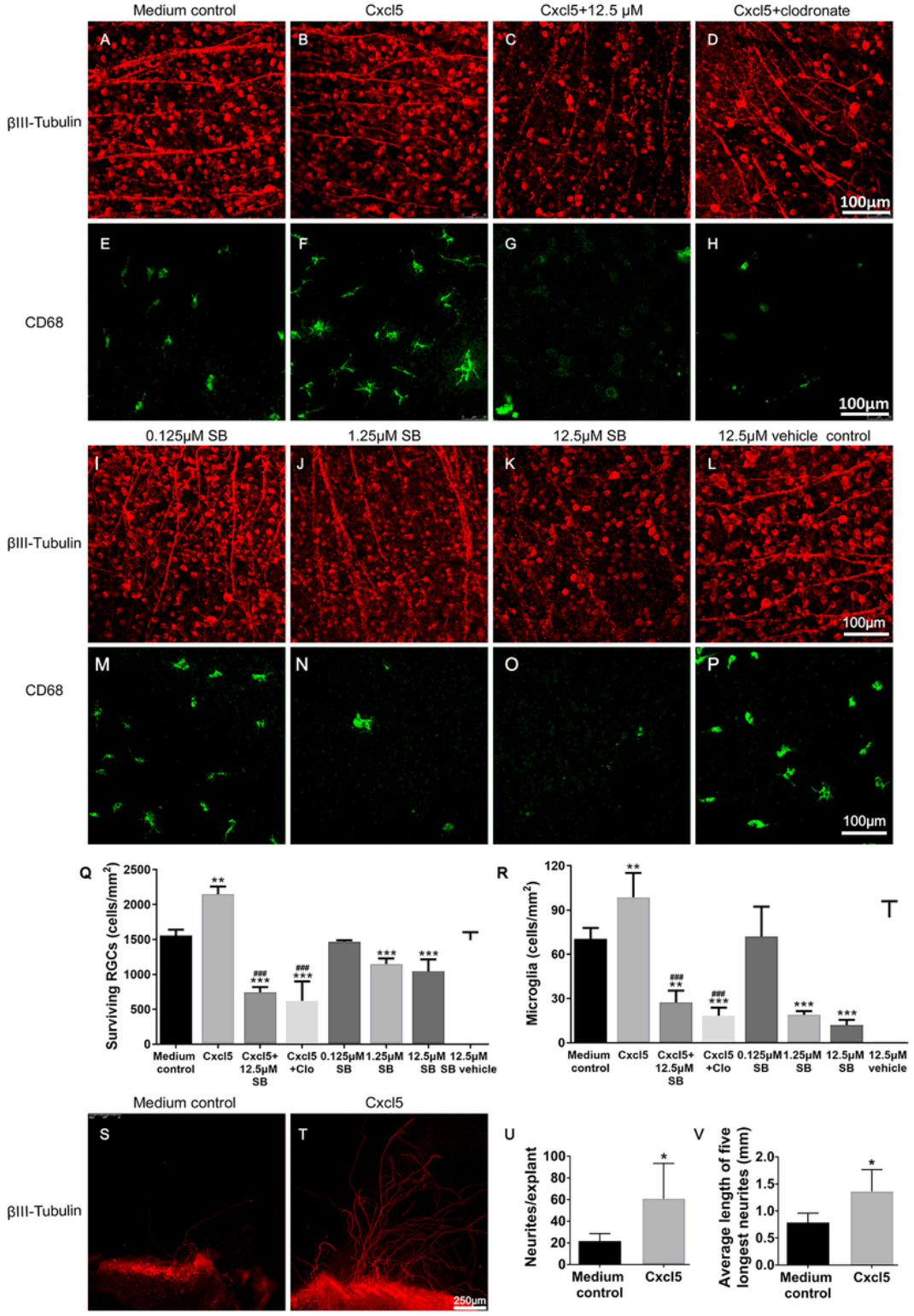

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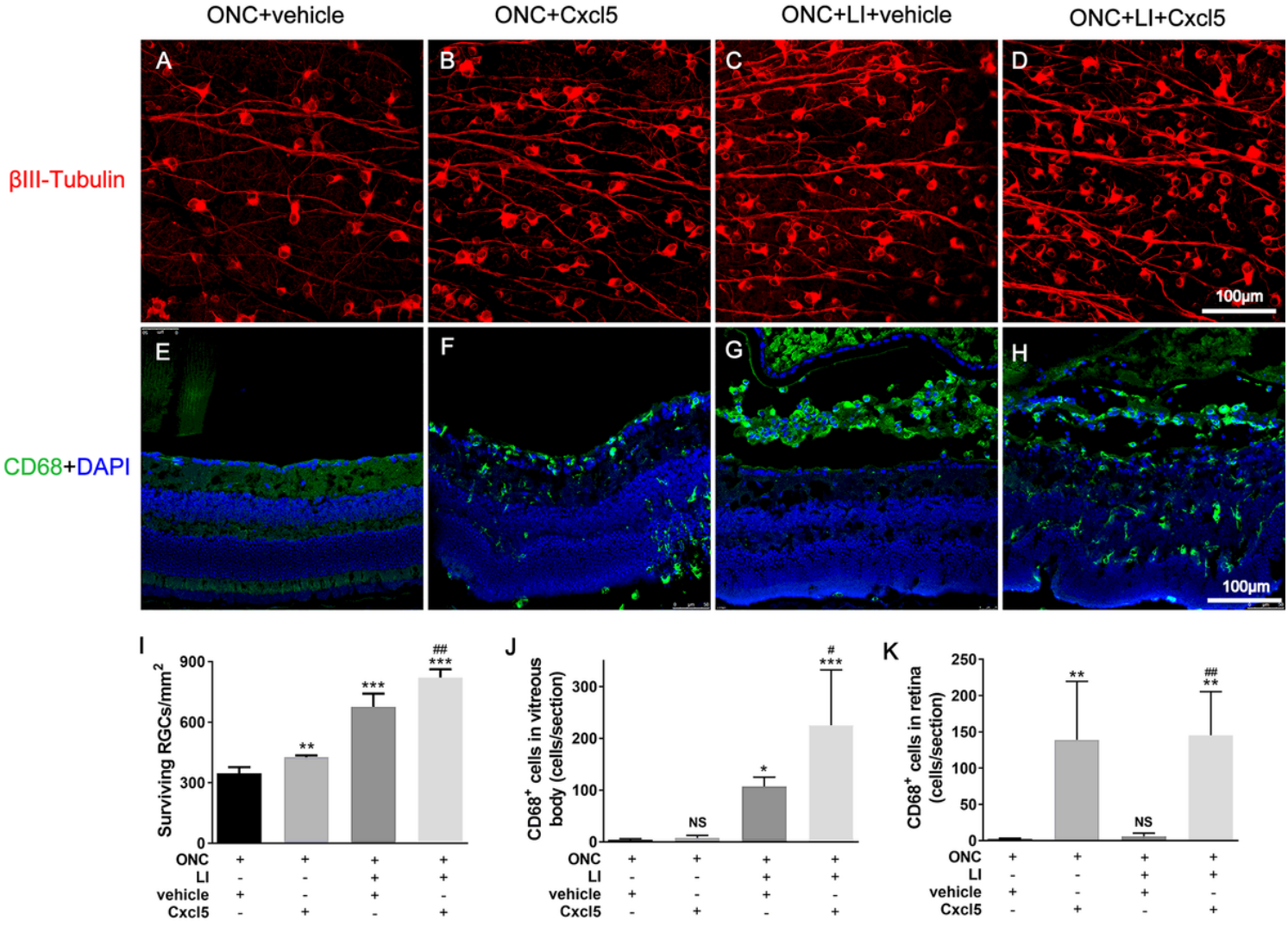
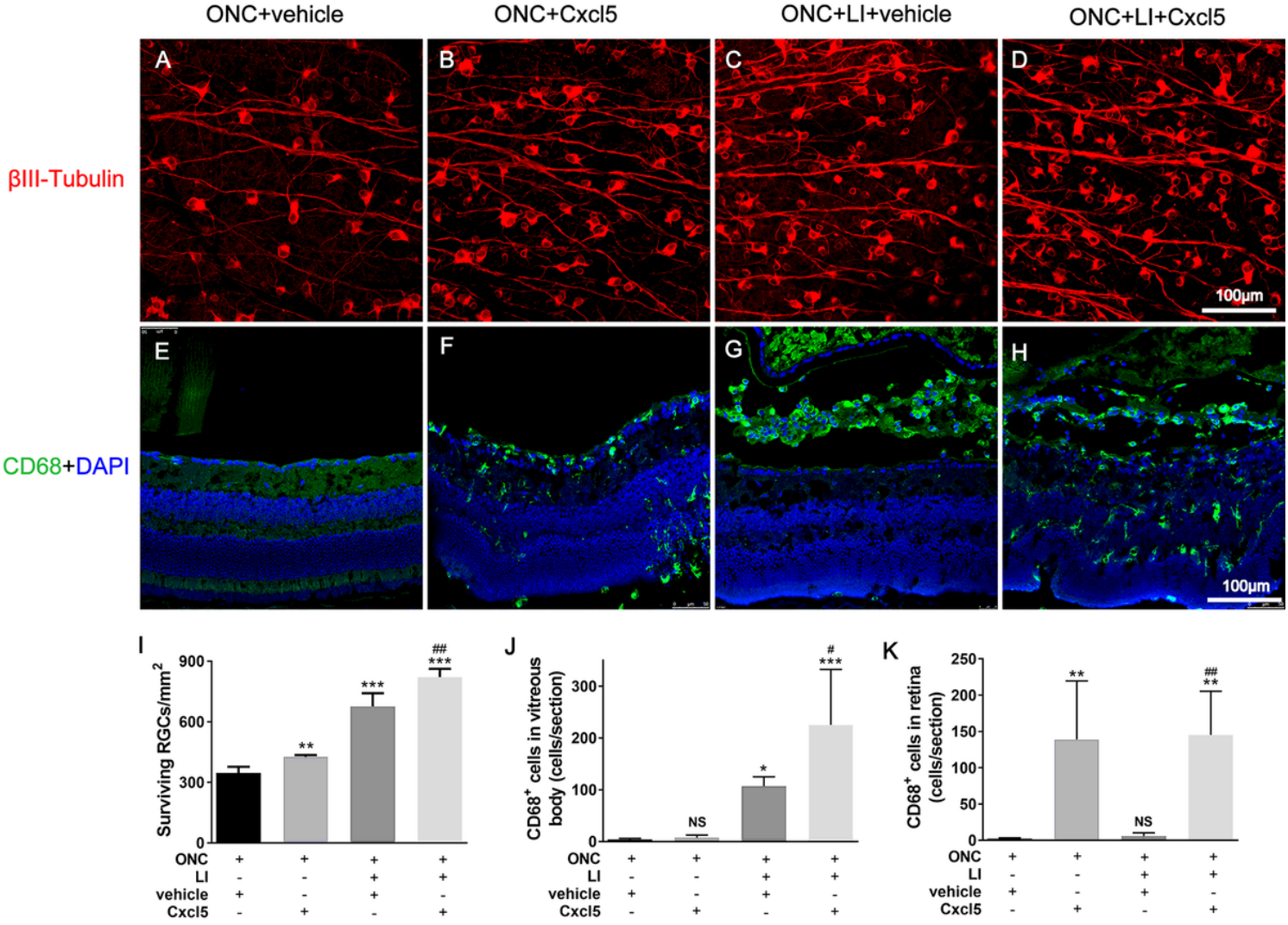


Figure 3

Cxcl5 promoted RGC survival after optic nerve injury and the protective effect of lens injury (A-D) Immunofluorescent staining images showed βIII tubulin+ RGCs in the whole mount retina 14 days after ONC or ONC+LI with or without Cxcl5 injection 3 and 7 days after surgery. (E-H) CD68+ inflammatory cell in the frozen retina section 7 days after ONC or ONC+LI with or without Cxcl5 injection 3 days after

surgery. Scale bar: 100 μm. (I) Average surviving RGCs, (J) Average CD68+ cells in vitreous per section and (K) Average CD68+ cells inside the retina in different groups.*: P < 0.05; **: P < 0.01; ***: P < 0.001, compare with ONC group. #: P < 0.05; ##: P < 0.01, compared with ONC+LI+vehicle group.



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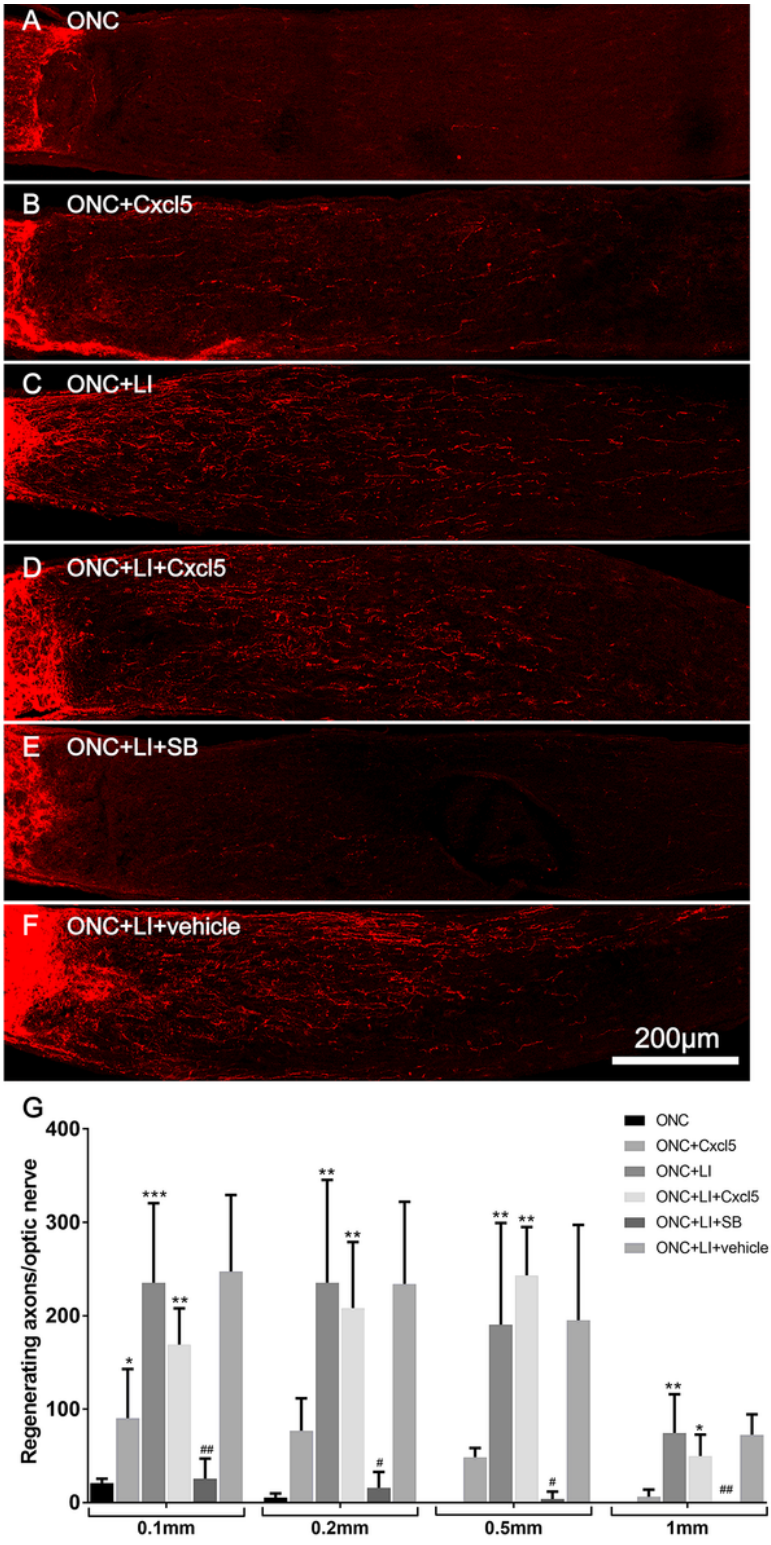
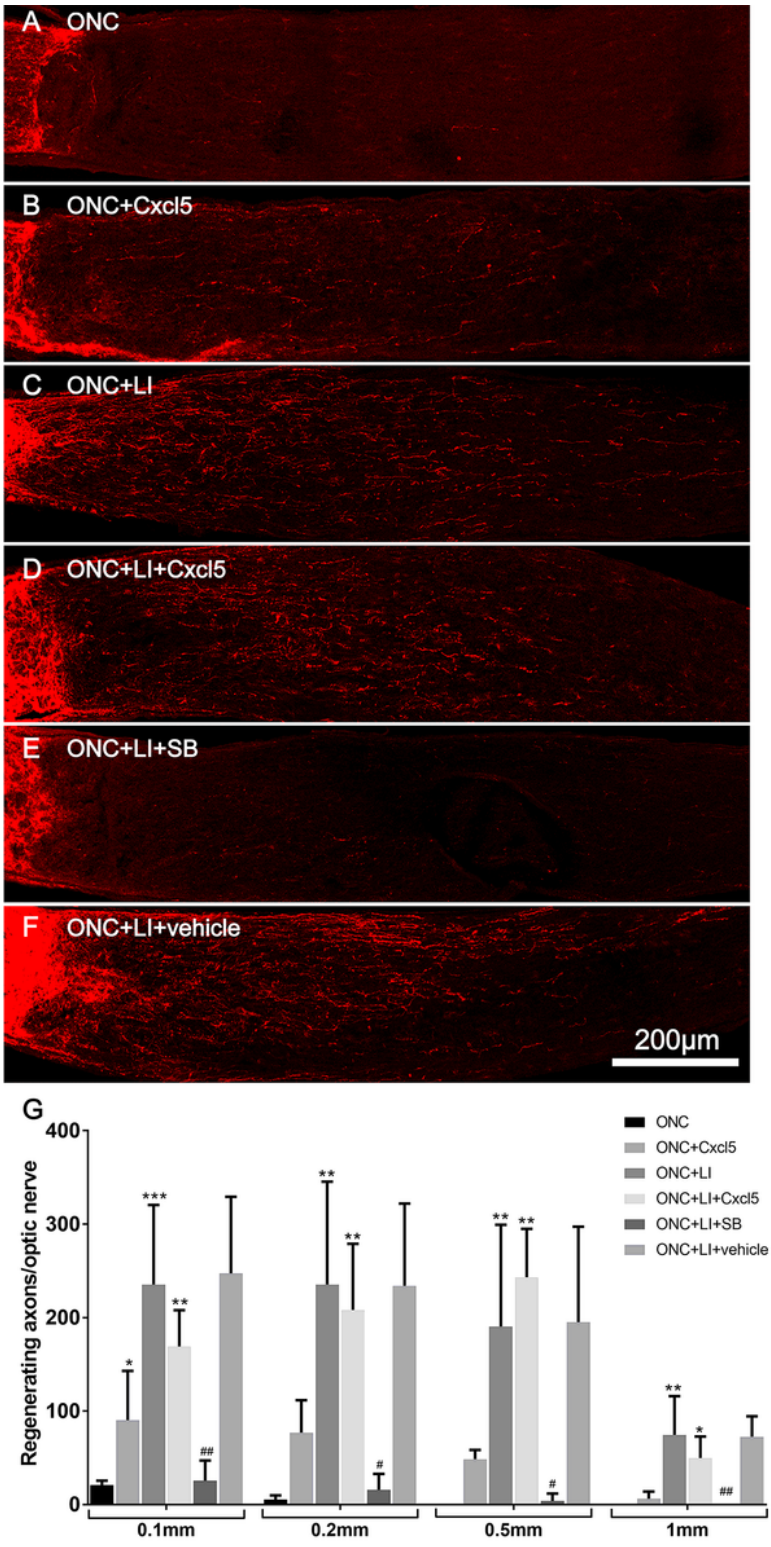


Figure 4

Axonal regeneration with the treatment of Cxcl5 and Cxcr2 antagonist after optic nerve injury (A-F) GAP43+ regenerated axons after ONC or ONC+LI with or without recombinant Cxcl5 and SB225002 injection. Scale bar: 200 μm. (G) Average regenerating axons at different distances. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$, compare with ONC group. #: $P < 0.05$; ##: $P < 0.01$, compare with ONC+LI+Cxcl5 group.



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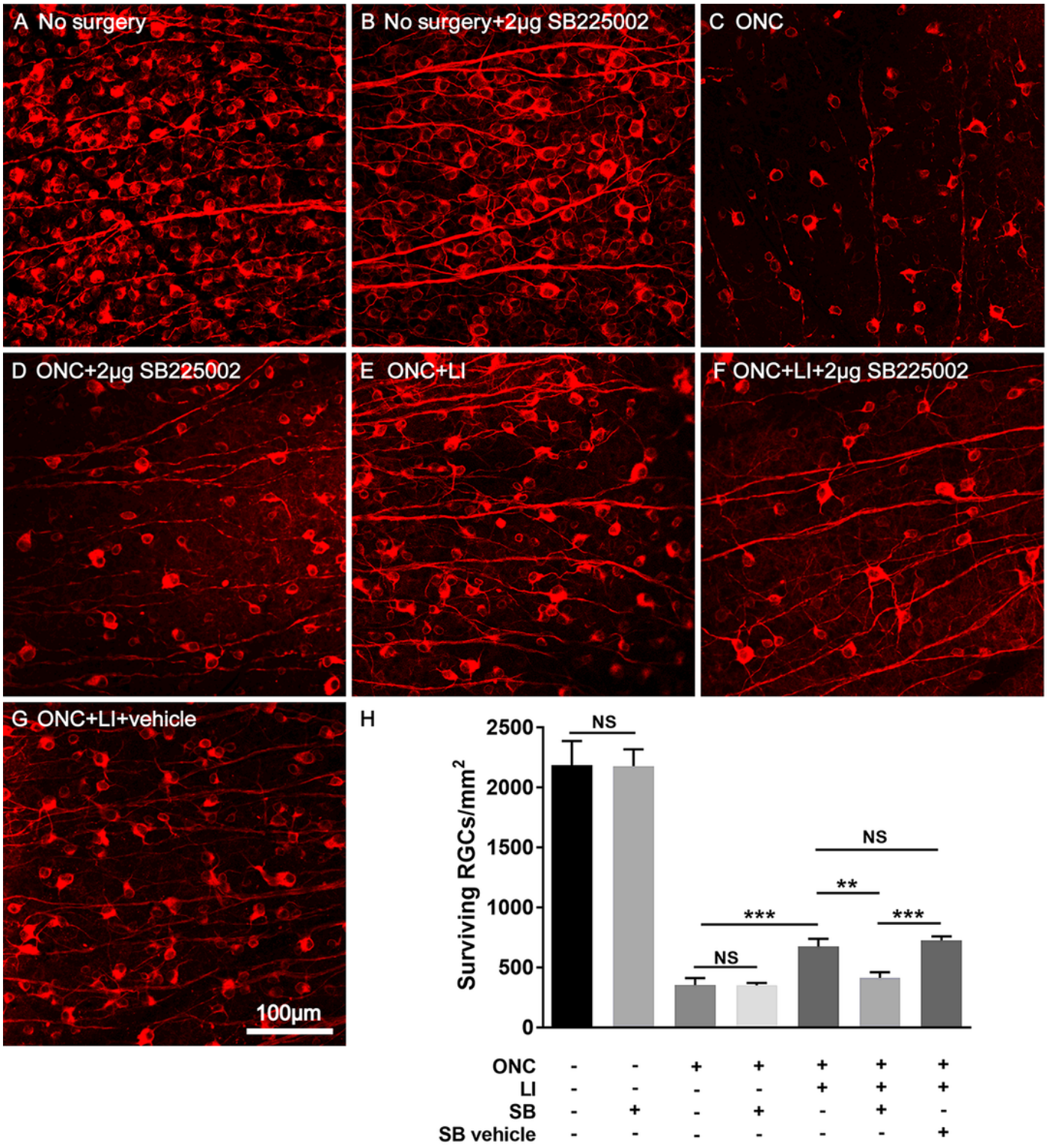
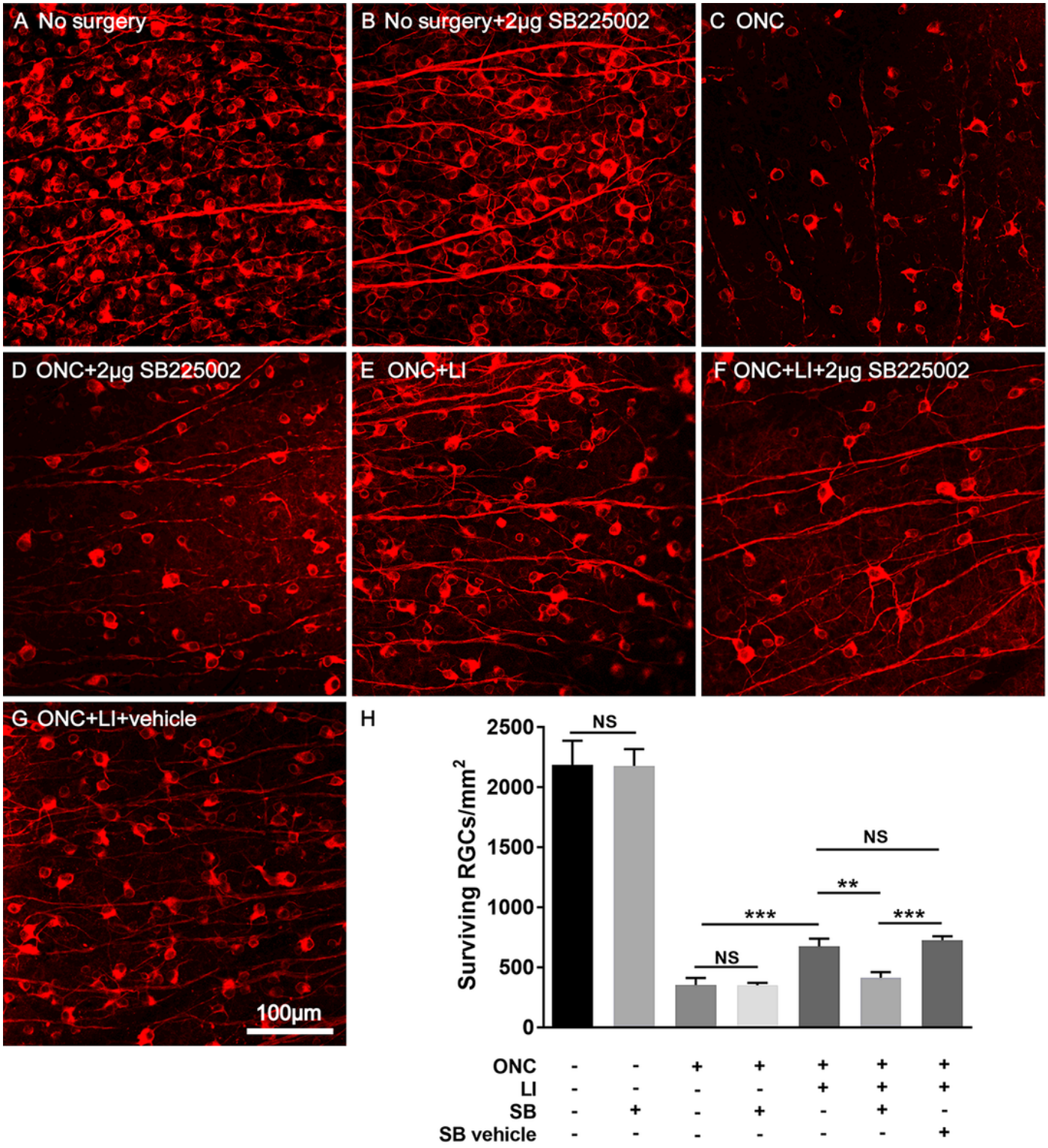


Figure 5

The effect of Cxcr2 antagonist on RGC survival after optic nerve and lens injury (A-G) Immunofluorescent staining images showed βIII tubulin+ RGCs in the whole mount retina in no surgery group, or 14 days after ONC or ONC+LI with or without intraperitoneal injection. Scale bar: 100 μm. (I) Average surviving RGCs in different condition. NS: $P > 0.05$; **: $P < 0.01$; ***: $P < 0.001$.



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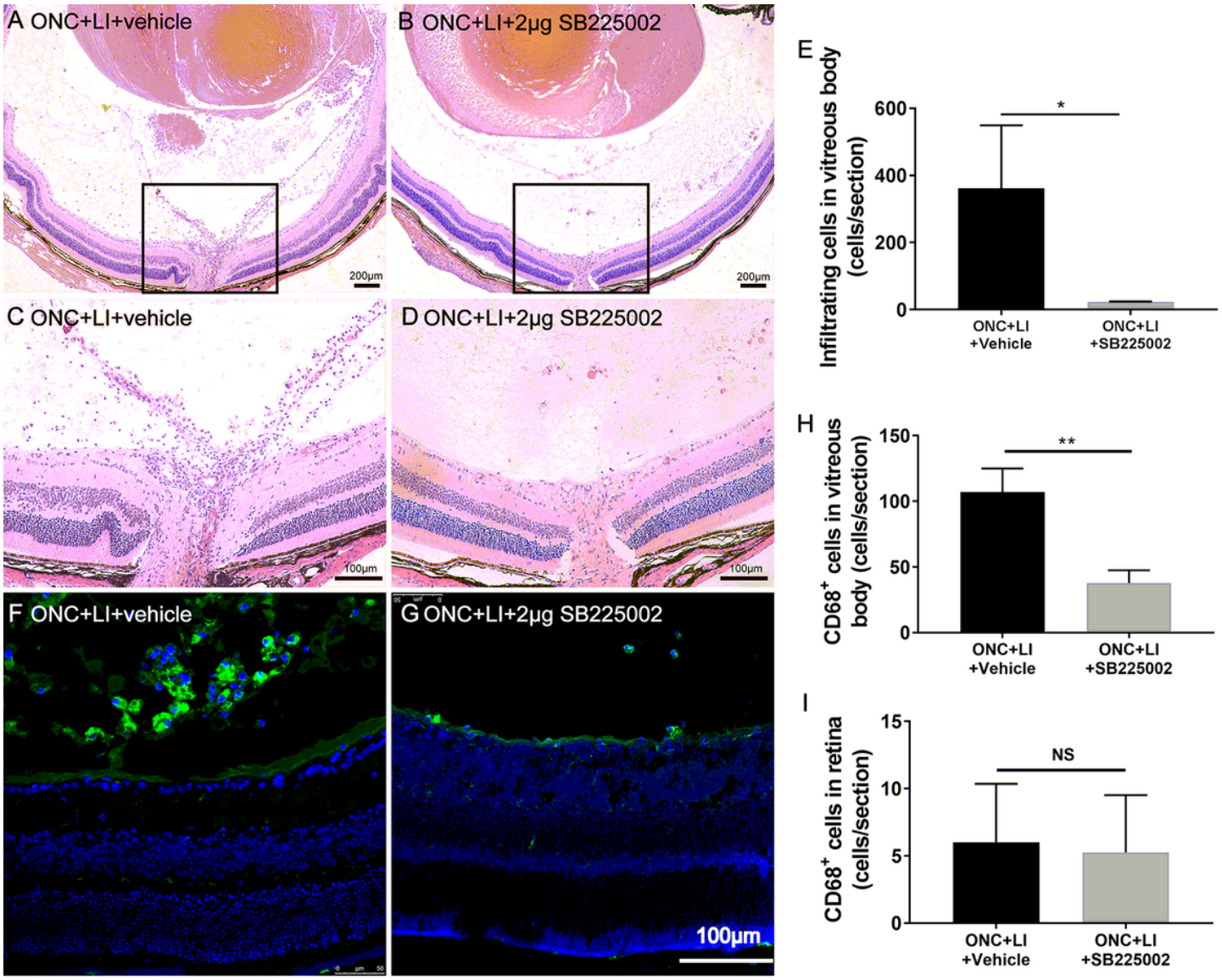
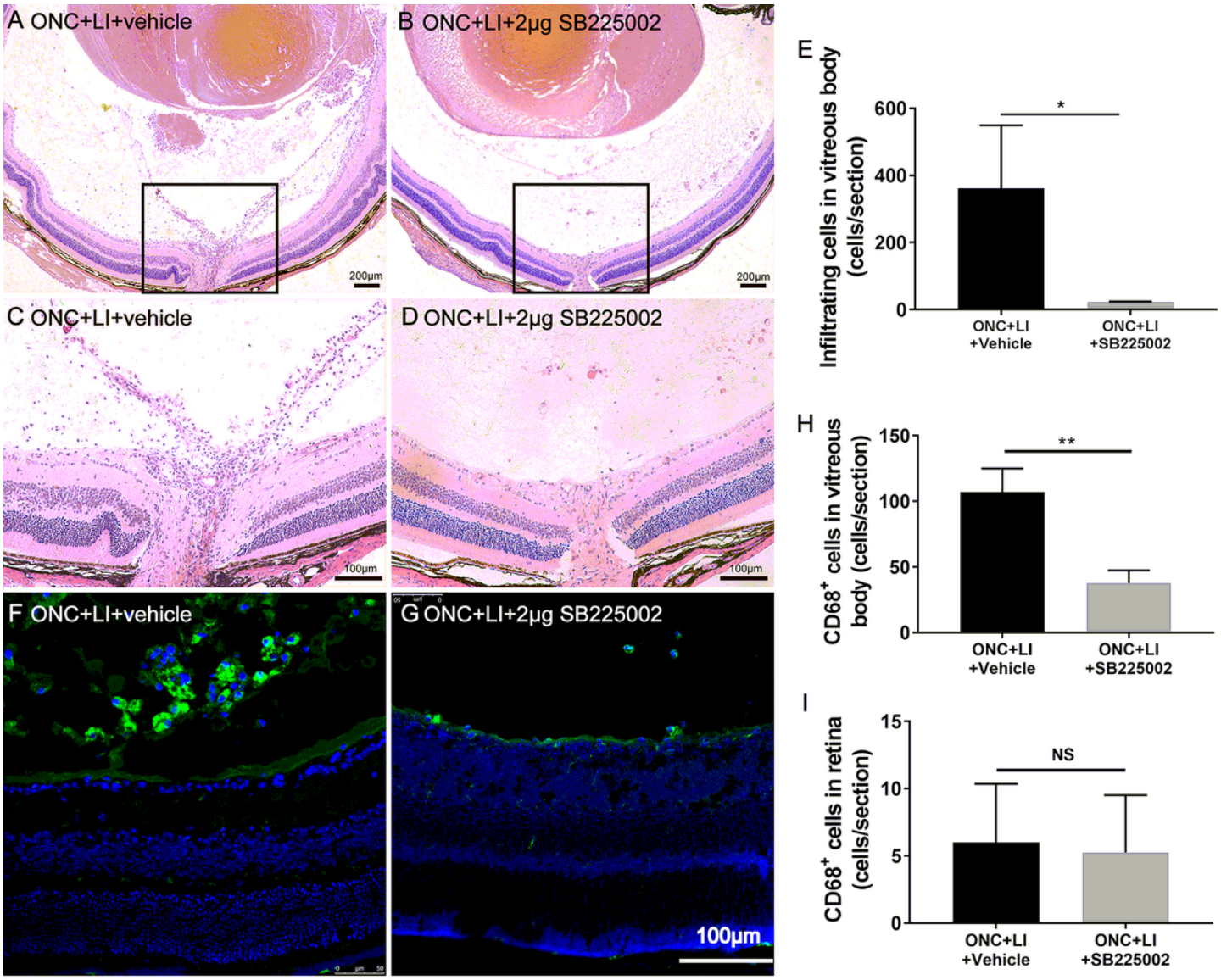


Figure 6

The effect of Cxcr2 antagonist on inflammatory responses after optic nerve and lens injury (A, B) Haematoxylin Eosinstaining showed that the infiltrating inflammatory cells 7 days after surgery with or without SB225002 injection. Scale bar: 200 μm. (C, D) Magnified images of optic nerve head area. Scale bar: 100 μm. (E) Average infiltrating cells in vitreous cavity. (F, G) Confocal immunofluorescent images showed CD68+ cells in frozen section, (H) average CD68+ cells in vitreous cavity, (I) and average CD68+ cells in retina.



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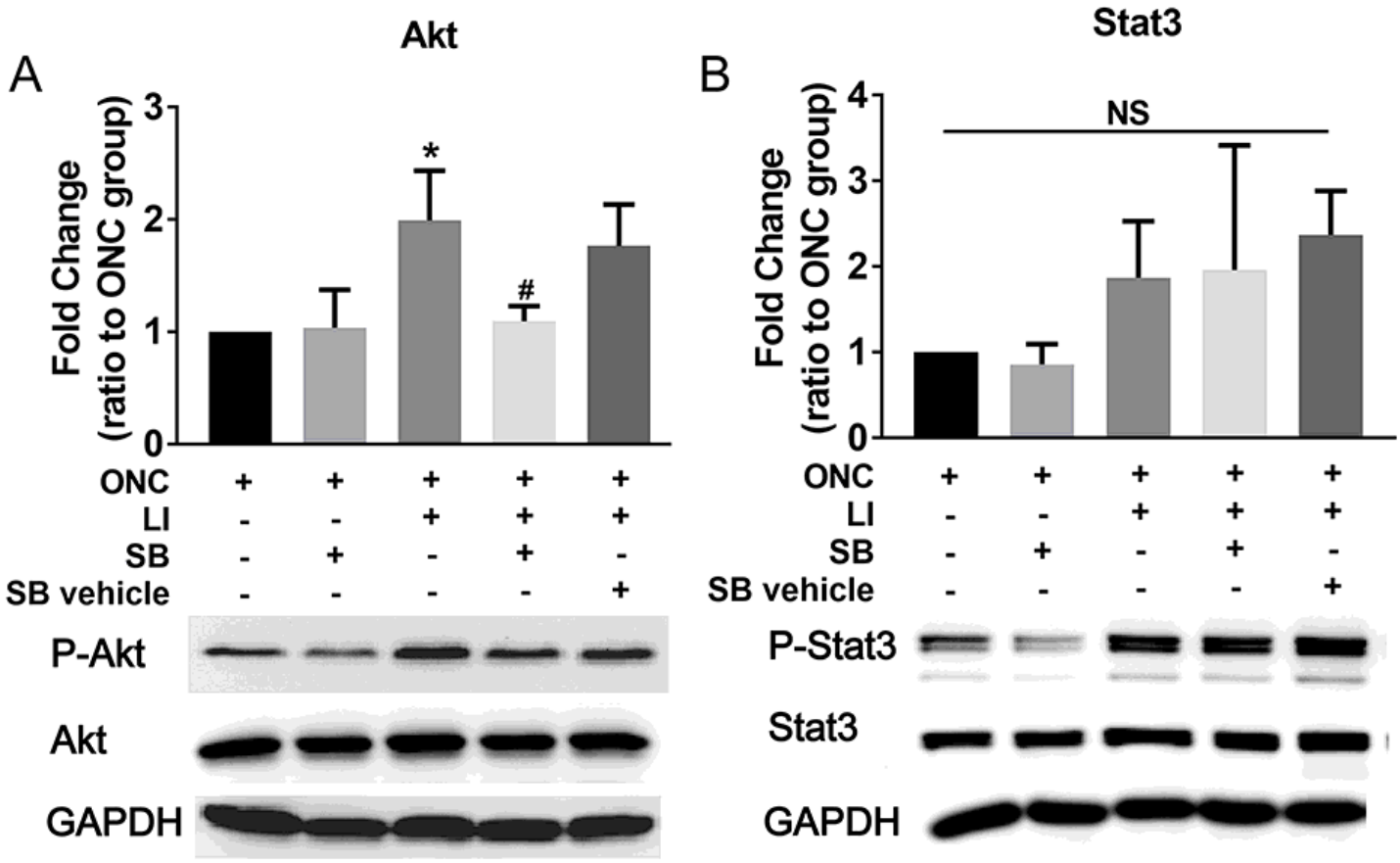
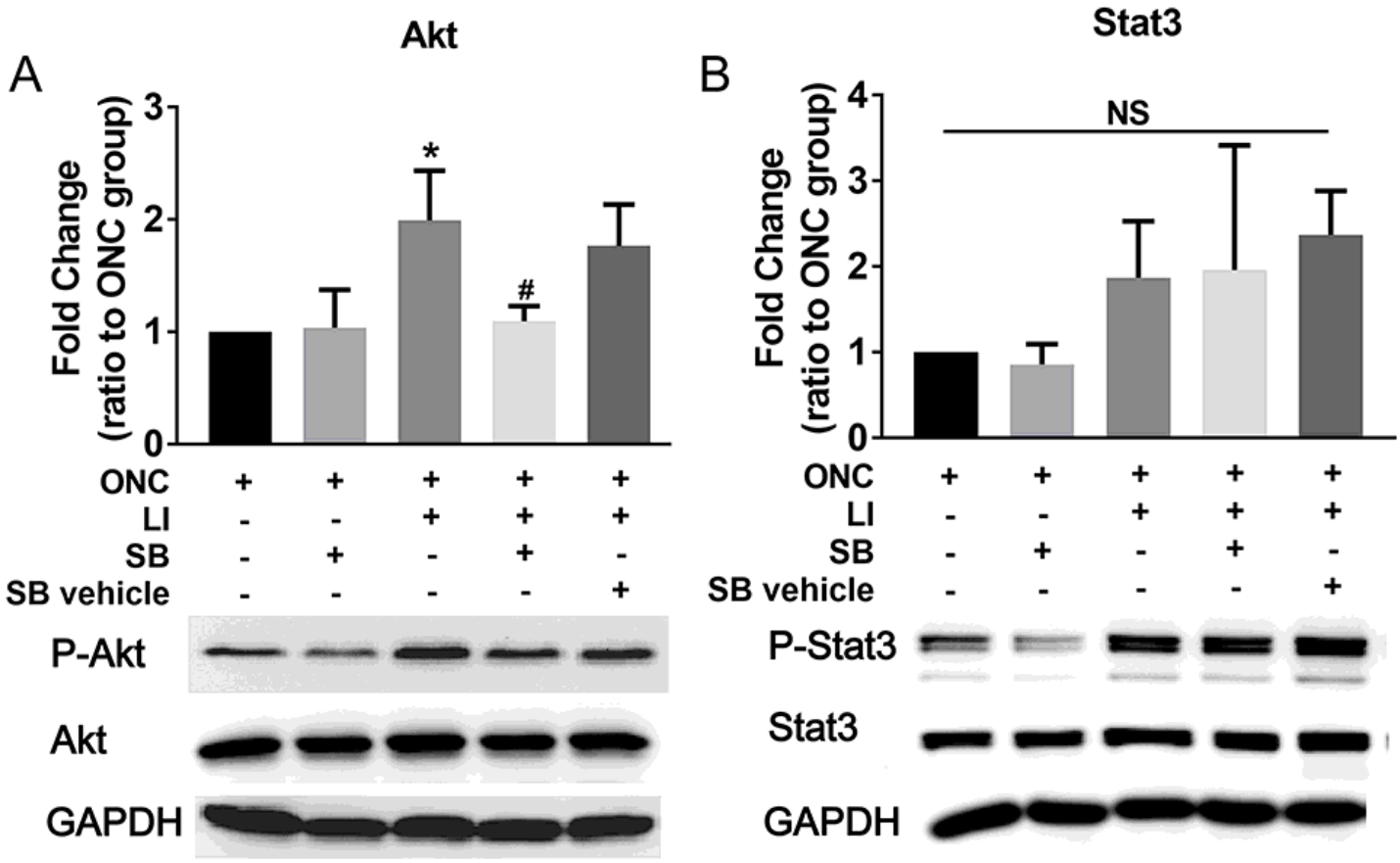


Figure 7

Akt and Stat3 activation in the retina of optic nerve and lens-injured mice after Cxcr2 antagonist treatment. Immunoblotting analysis of the Cxcr2 related signaling pathways in the retinas of SB225002 treated mice after ONC or ONC+LI. Fold changes and immunoblotting images of (A) phospho-Akt /Akt and (B) phospho-Stat3/Stat3. SB225002 attenuated LI activated phosphorylation of Akt, No obvious change was found in Stat3 pathway.*: $P < 0.05$, compare with ONC group, #: $P < 0.05$, compare with ONC+LI group.



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