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The levels and significance of inflammasomes in the mouse retina following optic nerve crush

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ABSTRACT

Inflammasomes play an important role in neuroinflammation. However, their function during the secondary death of retinal cells after traumatic optic neuropathy and their dependence on pathogen stimuli remains unclear. Therefore, we evaluated the expression profiles of 10 different inflammasome-related mRNAs in the retina following an optic nerve crush (OPC) injury under both conventional sterile as well as non-sterile conditions, and validated two significantly varied ones on a protein level. While most factors were much more highly elevated in non-sterile conditions, both Nlrp1b and Nlrp3 inflammasome mRNAs were increased significantly on postoperative day 1 to day 7 in the mouse sterile OPC injury model. While production of the inflammation-associated cytokines IL-1β and IL-18 could be continuously detected on an mRNA level postoperatively, a clear peak could be seen on day 7 that coincided with maximal expression of caspase-1 mRNA and with observation of retinal ganglion cells death, despite the mice being held in specific-pathogen free conditions. As such, the pro-inflammatory cytokines activated by inflammasome activation during OPC injury may drive secondary cell death through pyroptosis, and inhibition of these delayed responses may be an important means of preventing worsened injury and loss of vision in trauma patients.

1. Introduction

Optic nerve injury may be caused by a range of factors, including trauma, tumor, and glaucoma, and can result in an irreversible decrease in visual function or even complete vision loss. Neuroinflammation plays an important role in optic nerve injury, as the sustained amplification of activated immune response in response to the initial injury can cause secondary death of retinal ganglion cells. In cases of traumatic optic nerve injury, open wounds may be infected by bacterial/viral pathogens, leading to classical activation of the immune system to counter foreign entrants around the optic nerve; in contrast, a closed optic nerve injury can still induce sterile inflammation as a result of the release of damage-associated molecular patterns that may then trigger innate immune cell activation [1,2]. The causes of these two types of inflammation are quite different, and the sterile inflammatory response induced by closed optic neuropathy is more commonly observed in clinical settings.

A series of receptors known as pattern recognition receptors (PRRs) are involved in innate immune responses in the body by sensing various exogenous microbiological structures (i.e., pathogens and pathogen-associated molecular patterns) and endogenous danger signals (i.e. nucleic acid strands and other cellular debris). Ligation of these receptors initiates downstream signaling pathways that may trigger the production of pro-inflammatory cytokines and chemokines. The family of nucleotide-binding oligomerization domain-like receptors (NOD-like receptors, NLRs) and absent in melanoma 2-like receptors (ALRs) are prominent examples of PRRs. As intracellular sensor molecules, these receptors can recognize both endogenous or exogenous pathogens, as well as noninfectious factors [3,4]. In the presence of internal and external stimuli, members of the NLR and ALR families form polyprotein complexes known as inflammasomes. Inflammasomes are composed of the receptor protein apoptosis-associated speck-like protein containing CARD (ASC) and the effector molecule caspase-1 precursor (pro-caspase-1). Inflammasomes activate pro-caspase-1 through catalytic

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cleavage, triggering downstream cleavage of pro-IL-1β and pro-IL-18 into mature IL-β and IL-18 [5] that aggravate inflammatory responses in the surrounding tissues and potentially inducing cell death via pyroptosis [6]. Pyroptosis differs from conventional apoptosis, as apoptotic cells have intact cell membranes and do not stimulate immune reactions, whereas during pyroptosis, cells develop pores in the cell membranes through movement of factors such as gasdermin D, resulting in the leakage of cellular contents [7], which stimulate immunogenic reactions that cause sterile inflammation in tissues. Based on accumulating evidence, inflammasomes are closely associated with the development and progression of sterile inflammatory diseases, such as atherosclerosis, metabolic disease, and neurodegenerative [8] and neuroinflammatory diseases [9]. While recent studies have demonstrated that inflammasomes are also highly important in the eyes, the role of inflammasomes in inducing the sterile inflammatory response observed during traumatic optic neuropathy remains unclear. Nlrp3$^{-/-}$ mice display delayed retinal ganglion cells death associated with the attenuation of toxic side effects on the microglia following an optic nerve crush (OPC) injury [10]. In addition, an explosive injury model using mouse eyeballs [11] showed the expression of caspase-1, which is closely associated with inflammasomes, is induced in the retina [6]. Thus, inflammasomes might play an important role in optic nerve injury.

In this report, we screened mRNAs isolated from mouse retinas following OPC injury in both nonsterile and specific-pathogen free environments to identify inflammatory characteristics at different stages of the injury, and monitored changes in pyroptosis following the optic nerve injury, to determine which inflammasome proteins played substantial roles in the development and progression of diseases associated with OPC injury in mice. These findings will provide insights for further research and the clinical treatment of optic nerve injuries.

2. Materials and methods

2.1. Materials

Materials used in the present study included a reverse transcription reagent kit (TaKaRa RR047A, USA), a qPCR detection instrument (ABI VIIA@7, USA), a qPCR detection reagent kit (GoTap qPCR Master Mix A6001, Fushen Biotechnology, China), primers (Yingjun, China), an anti-caspase-1 (p20) mAb (Casper-1) (AdipoGen, Switzerland), an anti-IL-1β (OmnimAbs American), an anti-GAPDH Ab (Bioworld, USA), goat anti-rabbit IgG (H&L)-HRP (Bioworld, USA), goat anti-mouse IgG (H&L)-HRP (Bioworld, USA), an HRP system for detecting the primary antibody (SPlink Detection Kits, ZSGB-BIO, China), a TUNEL reagent kit (Roche, Switzerland), and a confocal microscope (Leica, Germany).

2.2. Methods

2.2.1. Animal grouping and model establishment

One hundred and eight 5–6-week-old, specific pathogen-free (SPF)-grade, healthy male mice with body weights ranging from 22 to 24 g were purchased from the Animal Center of the Research Institute of Surgery at Army Medical University. The mice were housed in a sterile environment with a constant temperature and humidity (SPF grade). All animal procedures conformed to the animal use and care guidelines of the National Institutes of Health of the USA (NIH Publications No. 8023, revised 1978). The present study was approved by the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University. Animals were divided into 6 groups: the normal group, the sham surgery group, and the postoperative day 1, 3, 7, and 14 groups. Each group included 6 animals. The eyeballs of the mice were examined and did not show any abnormalities. Establishment of the OPC injury model: Each mouse was administered the standard anesthetic dose of 50 mg/kg. After anesthetization was complete, the right eye of each mouse was used as the normal eye and the left eye was used as the surgical eye. After anesthetization, an incision was made in the temporal bulbar conjunctiva, and a backward blunt dissection of the fascia and muscle was performed. Fine cross tweezers were used to crush the optic nerve for 10 s at a site located approximately 2 mm behind the eyeball. After the surgery was complete, animals were housed for the indicated time points in either a general environment (nonsterile environment) or a sterile environment.

2.2.2. RT-PCR

Mice were sacrificed on postoperative days 1, 3, 7, or 14, and the retinas were collected. The expression levels of the Nlrp1b, Nlrp2, Nlrp3, Naip/Nlrc4, Nlrp5, Nlrp6, Nlrp12, and AIM2 mRNAs were detected using qRT-PCR (primers are listed in Table 1). Total RNA was extracted from tissues using TRIzol reagent. The cDNA templates were synthesized using the reverse transcription reagent kit (TaKaRa. USA). A quantitative PCR machine was used to amplify the cDNAs. Each sample was analyzed in 3 replicate wells. GAPDH was used as the internal control gene. The relative expression level of each mRNA was determined using the $2^{-\Delta\Delta Ct}$ method, where ΔΔCt = (Ct of the target gene in the model group − Ct of the internal control gene in the model

Table 1
Primer sequences for the target genes.

Gene Name	Forward	Reverse	Function
Pro-caspase-1	GAAGAACAGAACAAAGAAGATGGCACA	AGCTCCAACCCTCGGAGAAAGAT	inflammatory caspases, activated in different inflammasomes, cleaves pro-IL-1β and pro-IL-18 to mature forms and also mediated pyroptosis
Pro-IL-1β	TGGGCTGGACTGTTTCTA	ATCAGAGGCAAGGAGGAA	associated with inflammasomes and plays an important role in sterile and nonsterile inflammation
Pro-IL-18	AAGAAAGCCGCCTCAAAC	GATTCCAGGTCTCCATTT	
HMGB1	TTGGGTCACATGGATTATTAGTGT	CAGGGCATGTGGACAAAAG	
Pro-caspase-3	CTAATCTGACGGTCCTCC	TCGCCAAATCTTGCTAAT	apoptotic effector (executioner) caspase
RIPK3	CAGTGGGACTTCGTGTCCG	CAAGCTGTGTAGGTAGCACATC	mediates necroptosis
PARP1	GGCAGCCTGATGTTGAGGT	GCGTACTCCGCTAAAAAGTCAC	the product of PARP activity, PAR, results in parthanatos
GPX4	GATGGAGCCCATTCCTGAACC	CCCTGTACTTATCCAGGCAGA	inhibitors of GPX4 trigger ferroptosis
GAPDH	CCTGGTATGACAATGAATACGGC	CTCCTTGGAGGCCATGTAGG	reference gene
Nlrp1b	TAGAAACGCCAGATAGGGTGA	AGTGTGATGGAAGTAATGGGGAT	These proteins form inflammasomes, which are polyprotein complexes. Their activation is important for the induction of sterile inflammation and nonsterile inflammation in the presence of internal and external stimuli
Nlrp2	TCTCATGTGCCTTCTACATCAGC	CACAAAGGTCACGGATCAGAG	
Nlrp3	GTGAAACAAAACGTGCCTTAGAA	GGAGGGCTTGATAGCAGTGAA	
Nlrp5	GAAAGCACAATGGGTCCTCCA	CTGACGCCTGTTCCACTTCT	
Nlrp6	TCTCTCCGTGTCAGCGTTCA	CGGAAGAGCCGATTAAAAGTGT	
Nlrp12	CTCTCAGCACCTTTCAGAGGA	GTGCATTGCGGTCTTCCATTA	
AIM2	TACCGGGAAATGCTGTTGTTG	AGTGTGCTCCTGGCAATCTGA	
Nlrc4	TTGAAGGCGAGTCTGGCAAAG	TTGAAGGCGAGTCTGGCAAAG	
Naip5	TGCCAAACCTACAAGAGCTGA	CAAGCGTTTAGACTGGGGATG	
Naip6	TACAGGGAGTTTACAAGACCCC	AGTGGCCTGGAGAGACTCAG	

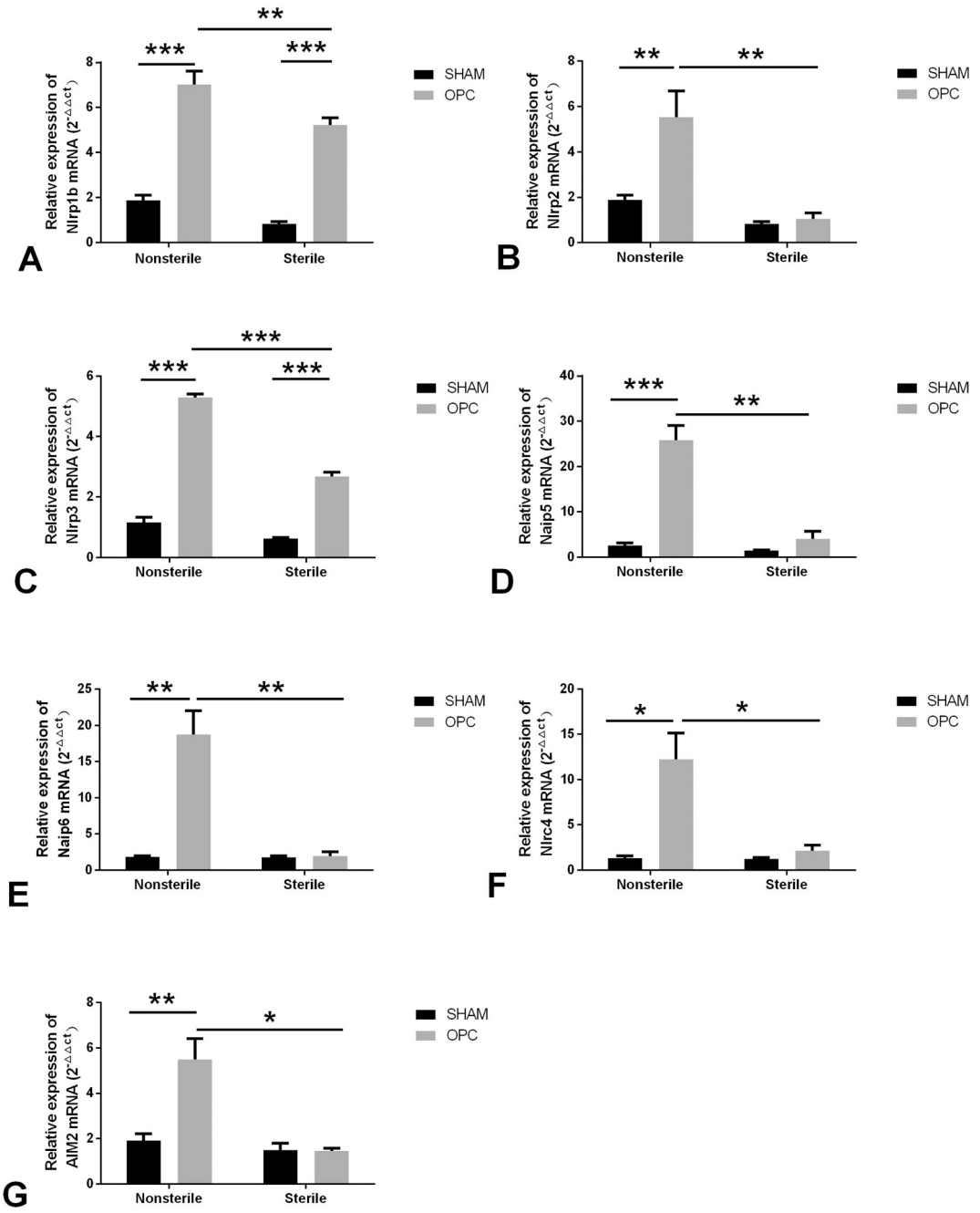


Fig. 1. The expression of the Nlrp1b and Nlrp3 mRNAs was significantly increased under both the sterile and nonsterile conditions, while the levels of the Nlrp2, AIM2, and Naip/Nlrc4 mRNAs were increased only under the nonsterile condition. The expression of different inflammasome genes was detected using qPCR on postoperative day 7 in retinas from mice in the surgery and sham surgery groups that were maintained under sterile and nonsterile conditions following OPC injury. A: Expression of the Nlrp1b mRNA on postoperative day 7. B: Expression of the Nlrp2 mRNA on postoperative day 7. C: Expression of the Nlrp3 mRNA on postoperative day 7. D: Expression of the Naip5 mRNA on postoperative day 7. E: Expression of the Naip6 mRNA on postoperative day 7. F: Expression of the Nlrc4 mRNA on postoperative day 7. G: Expression of the AIM2 mRNA on postoperative day 7. Data were analyzed using independent sample *t*-tests. All results are presented as the mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

group) − (Ct of the target gene in the normal control group − Ct of the internal control gene in the normal control group). Primer sequences for the target genes are listed in Table 1.

2.2.3. Western blot

Mouse retinas were separately collected and lysed with RIPA buffer to extract the total proteins. For each group, triplicate proteins samples were prepared from independent retinal proteins samples, each of which was derived from the retinas of 3 different rats. Proteins were electrophoretically separated on 15% SDS-polyacrylamide gels and then transferred to PVDF membranes. Membranes were blocked with 5% skim milk at room temperature for 2 h, incubated with primary antibodies specific for cleaved caspase-1 (P20) (1:1000), cleaved IL-1β (1:500), or GAPDH (1:5000) overnight at 4 °C, then incubated with the appropriate secondary antibody (1:5000) for 1.5 h at room temperature, and signals were developed using enhanced chemiluminescence (ECL). Membranes were exposed to film, and the films were scanned and analyzed using ImageJ software.

2.2.4. Immunohistochemistry

Specimen collection and processing: the eyes were harvested at the end of the experiment and immediately fixed with 4% paraformaldehyde overnight. The specimens were subsequently dehydrated in a graded series of ethanol solutions, embedded in paraffin and cut into 4 μm sections. Before deparaffinization, the sections were baked in a 60 °C oven for 2 h, rehydrated with an ethanol gradient and placed into 0.01 mol/L (pH 6.0) citric acid buffer for antigen retrieval. Slices were incubated with 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity and washed with phosphate-buffered saline (PBS). After preincubation with goat serum for 30 min at room temperature to block nonspecific binding, sections were incubated with the primary antibody (AG-20B-0042, Adipogen, Switzerland), which could recognize both full-length and cleaved (p20 fragments) forms of mouse caspase-1 and differentiate between the two based on band size, overnight at 4 °C. Membranes were then warmed at 37 °C for 1 h and washed with PBS. An HRP detection system for the primary antibody was used according to the manufacturer's instructions. HRP activity was revealed using diaminobenzidine (DAB). All sections were lightly counterstained with hematoxylin. Sections were observed under a light microscope, and at least random 6 sections per group were used for pro-caspase-1 and cleaved caspase-1 (p20) immunohistochemistry.

2.2.5. TUNEL fluorescence staining

Paraffin sections were selected, baked in a 60 °C oven for 2 h, rehydrated with an ethanol gradient and placed in 0.01 mol/L (pH 6.0) citric acid buffer for antigen retrieval. Sections were permeabilized with a 0.1% citric acid solution containing 0.1% Triton X-100 at room temperature for 15 min. After rinses with PBS, the TUNEL reaction mixture solution was prepared at a 1:9 ratio, according to the manufacturer's instructions and was used to cover the positive control group. The negative control group was incubated with only the dUTP solution. The reaction was performed at 37 °C in the dark for 45 min. Sections were stained with DAPI and mounted. The results were observed under a fluorescence microscope. Positive cells were counted using ImageJ software.

2.2.6. Statistical methods

Experimental data were analyzed using SPSS 19.0 statistical software. After confirming a normal distribution with a statistical test for skewness and kurtosis, independent sample *t*-tests were used to analyze differences between two groups, or one-way ANOVA or two-way repeated-measures ANOVA followed by the least significant difference (LSD) post hoc test was used to analyze differences between multiple groups. Data are presented as the mean ± standard deviation (mean ± sd). $P < 0.05$, $P < 0.01$, or $P < 0.001$ indicated significant differences.

3. Results

3.1. The expression of inflammasome genes in mouse retinas following an OPC injury was significantly different between animals housed in nonsterile and sterile environments

Inflammasomes are activated by pathogen-associated molecule patterns (PAMPs) and damage-associated molecule patterns (DAMPs). In this study, we performed qPCR of RNA extracted from the retinas of mice with an OPC injury after housing for 1, 3, 7, and 14 d under nonsterile and sterile conditions. The expression of the Nlrp1b, Nlrp2, Nlrp3, AIM2, and Naip/Nlrc4 mRNAs differed between the two conditions, while the expression of the Nlrp5, Nlrp6, and Nlrp12 mRNAs was not detectable. In addition, the highest levels of the majority of inflammasome-associated mRNAs were observed on postoperative day 7. The levels of the Nlrp2, AIM2, and Naip/Nlrc4 mRNAs present in retinas from mice housed under the nonsterile condition were significantly increased on day 7, while no obvious increases in the levels of these transcript were observed in retinas from mice housed under sterile conditions (Fig. 1B, D, E, F and G). However, the levels of the Nlrp3 and Nlrp1b mRNAs were significantly increased under both conditions (Fig. 1A and C). These results suggested to us that a portion of inflammasome activity during optic nerve crush is indeed delayed, and likely stimulated by secondary activation of immune cells instead of being directly stimulated by the primary trauma event.

3.2. The inflammasome genes Nlrp1b and Nlrp3 were differentially expressed in the mouse retina following OPC injury and housing in sterile conditions

On postoperative days 1, 3, 7, and 14, the retinal tissues from mice in the surgery and the control group (sham surgery group) that were maintained in the sterile environment were collected for qPCR to further confirm the expression of the inflammasome genes Nlrp1b and Nlrp3 in the retinas of mice with an OPC injury that were maintained under sterile conditions. The expression of the Nlrp1b and Nlrp3 genes differed at different time points after OPC injury (Fig. 2A, B). Nlrp1b was expressed at high levels on postoperative days 3 and 7, with the highest expression level detected on postoperative day 7, whereas Nlrp3 expression was detected on postoperative days 1, 3, and 7, with the highest expression level observed on postoperative day 3.

3.3. Comparison of the genes or proteins related to different forms of cell death in the retina following OPC injury in mice

The expression of genes or proteins encoding key enzymes and related to different cell death pathways, including pyroptosis, apoptosis, necroptosis, ferroptosis, and parthanatos, were detected in mouse retinas 1, 3, 7, and 14 d after OPC injury using qPCR or Western blotting, respectively, to confirm whether diverse cell death pathways were activated during traumatic optic neuropathy. Compared to the sham surgery group, the peak expression of the abovementioned genes and proteins was not detected to follow similar kinetics, suggesting that these processes may be initiated and terminated in response to different stimuli. The levels of the pro-caspase-1 and pro-caspase-3 mRNAs increased to different extents on postoperative days 3, 7, and 14, with pro-caspase-1 expression peaking on postoperative day 7 and pro-caspase-3 expression peaking on postoperative day 3 (Fig. 3A, D). These mRNA changes could also be seen on a protein level in the case of pro-caspase-1 (Fig. 3B). The expression of RIPK3 mRNA increased on postoperative days 1, 3, and 7, with peak expression occurring on postoperative day 3 (Fig. 3C). On the other hand, the expression of the PARP-1 and GPX4 mRNAs exhibited only a slight increase in the surgery group during the postoperative period, but the difference was not significant.

3.4. Expression of the inflammasome-associated inflammatory factors IL-1β, HMGB1, and IL-18 after mouse OPC injury and housing under sterile conditions

The activation of inflammasomes results in the production of the inflammation-associated cytokines pro-IL-1β and pro-IL-18 and the active secretion of HMGB1 [12]. Therefore, levels of the pro-IL-1β, pro-IL-18, and HMGB1 mRNAs were detected using qPCR in mouse retinal tissues 1, 3, 7, and 14 d after OPC injury in the sterile environment. The levels of the IL-1β mRNA and protein were increased at different times after surgery, with the highest levels observed on postoperative day 7 (Fig. 4A, B). The levels of the HMGB1 mRNA started to increase on postoperative day 3 (Fig. 4C), but the levels of the pro-IL-18 mRNA immediately started to increase on postoperative day 1 and peaked on postoperative day 14 (Fig. 4D).

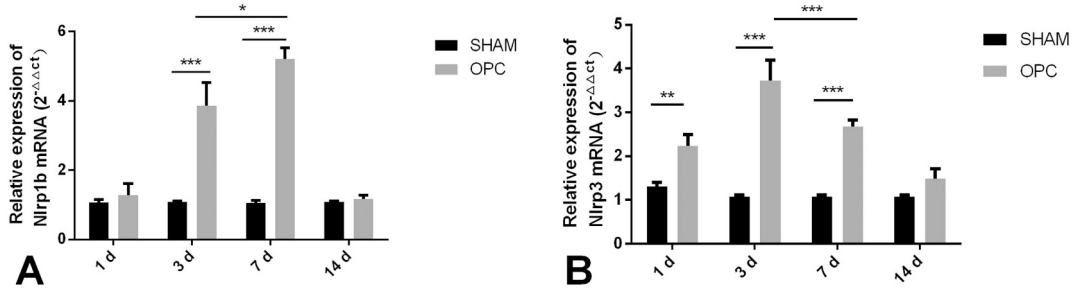


Fig. 2. The peak expression of the inflammasome gene Nlrp1b was observed on postoperative day 7 in mouse retinas following OPC injury and housing in sterile conditions, whereas Nlrp3 peaked on postoperative day 3. A: The expression of the Nlrp1b mRNA in retinas harvested at different time points after OPC injury in mice from the surgery and the sham surgery groups housed under sterile conditions was detected using qPCR. B: The expression of the Nlrp3 mRNA in retinas harvested at different time points after OPC injury in mice from the surgery and the sham surgery groups housed under sterile conditions was detected using qPCR; Data were analyzed using two-way repeated-measures ANOVA. All results are presented as mean ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

3.5. Peak retinal ganglion cells death coincides with high caspase-1 levels

In order to confirm that the changes in expression profiles we observed was indeed spatiotemporally significant, we then performed TUNEL immunohistochemistry was performed on retinal sections at postoperative days 1, 3, 7, and 14 following OPC injury in the sterile environment. The results showed an increase in the number of TUNEL-positive retinal ganglion cells in surgery groups throughout the experiment (Fig. 5A), with the peak stage of retinal ganglion cells death occurring on postoperative day 7, which reached approximately 60% of the cells. Immunohistochemical staining of the retina at this time point confirmed that high levels of pro-caspase-1 and cleaved caspase-1 (p20) could also be detected in the retinal ganglion cells layer (Fig. 5B). These results confirm that the ganglion cell death observed following OPC injury was indeed delayed and potentially linked to inflammasome activation of caspase-1.

4. Discussion

After optic nerve injury, the expression of inflammasome genes that were induced under sterile and nonsterile conditions differed. The expression of the inflammasome genes Nlrp1b and Nlrp3 resulted in sterile inflammation during closed optic neuropathy.

Nlrp1b [13] and Nlrp3 [14] have been reported to be activated by PAMPs and injury-associated molecular patterns under different inflammasome activation conditions; Naip/Nlrc4 [15] are activated by flagellin and type III secretory system proteins, and AIM2 [6] is activated by pathogens and DNA. We compared the mRNA expression

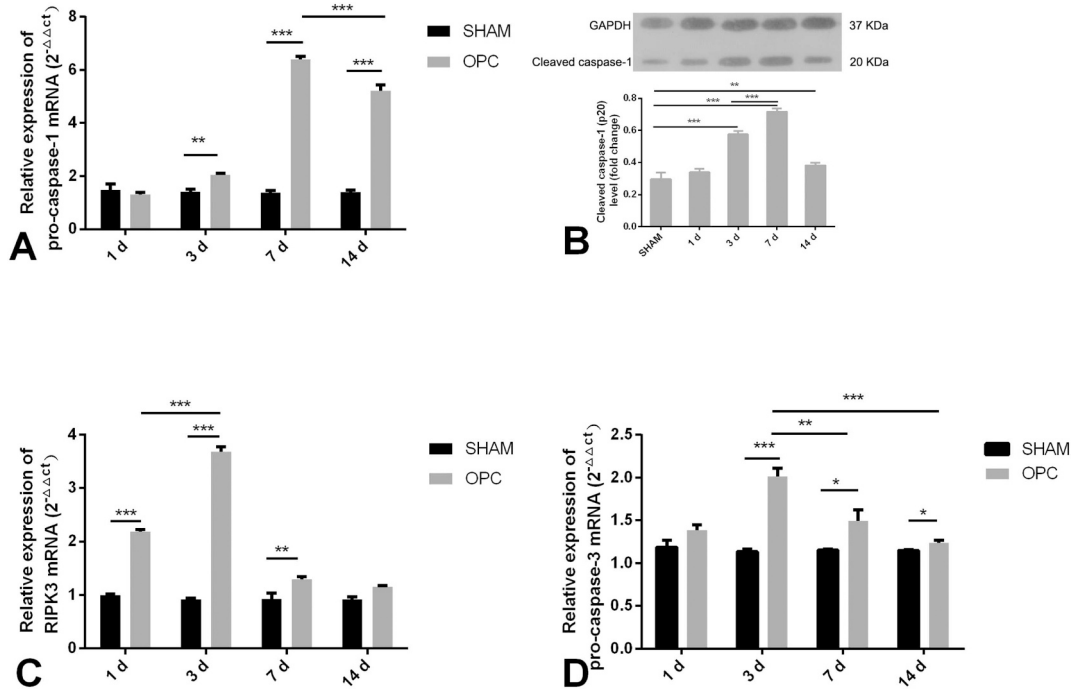


Fig. 3. In the mice with an OPC injury that were housed under sterile conditions, the highest levels of the pro-caspase-1 mRNA and the cleaved caspase-1 (p20) protein were detected on day 7, whereas the peak expression of the pro-caspase-3 and RIPK3 mRNAs was detected on postoperative day 3. A: The expression of the pro-caspase-1 mRNA was assessed in the mouse retinas from the surgery and sham surgery groups at different points after OPC injury and housing under sterile conditions using qPCR. B: The levels of the cleaved caspase-1 (p20) protein in the mouse retinas from the surgery and sham surgery groups were detected at different time points after OPC injury and housing under sterile conditions using Western blotting. C: The expression of the RIPK3 mRNA in the mouse retinas from the surgery and sham surgery groups was detected at different time points after OPC injury and housing under sterile conditions using qPCR. D: The levels of the pro-caspase-3 mRNA in the mouse retinas from the surgery and sham surgery groups were detected at different time points after OPC injury and housing under sterile conditions using qPCR. The qPCR data were analyzed using two-way repeated-measures ANOVA and the Western blot data were analyzed using one-way ANOVA. All results are presented as mean ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

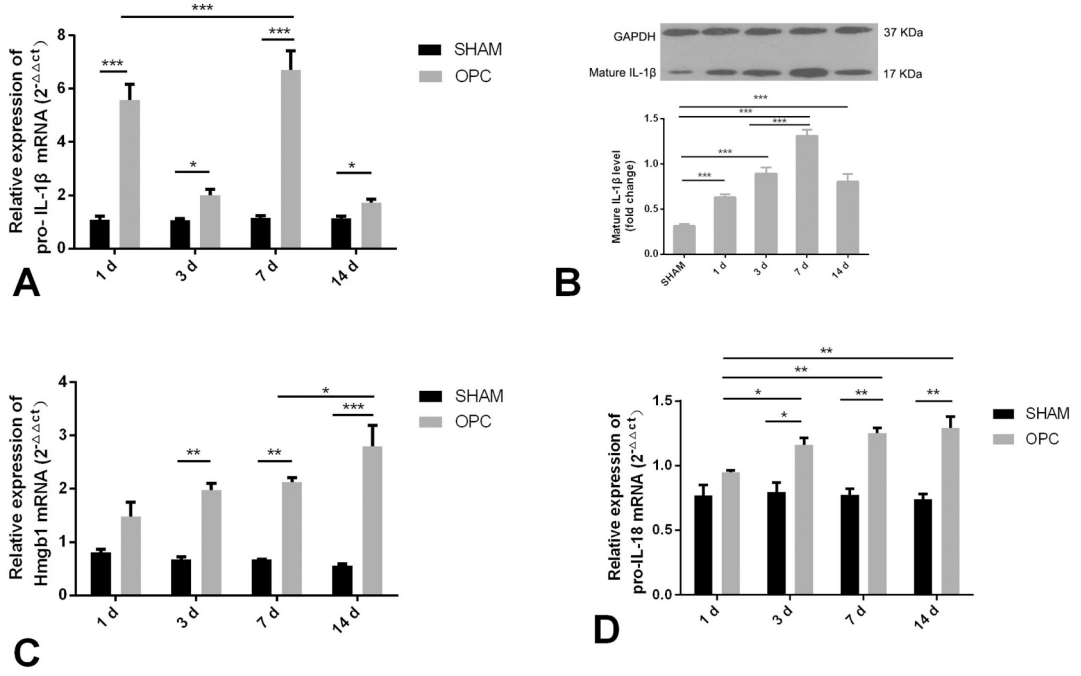
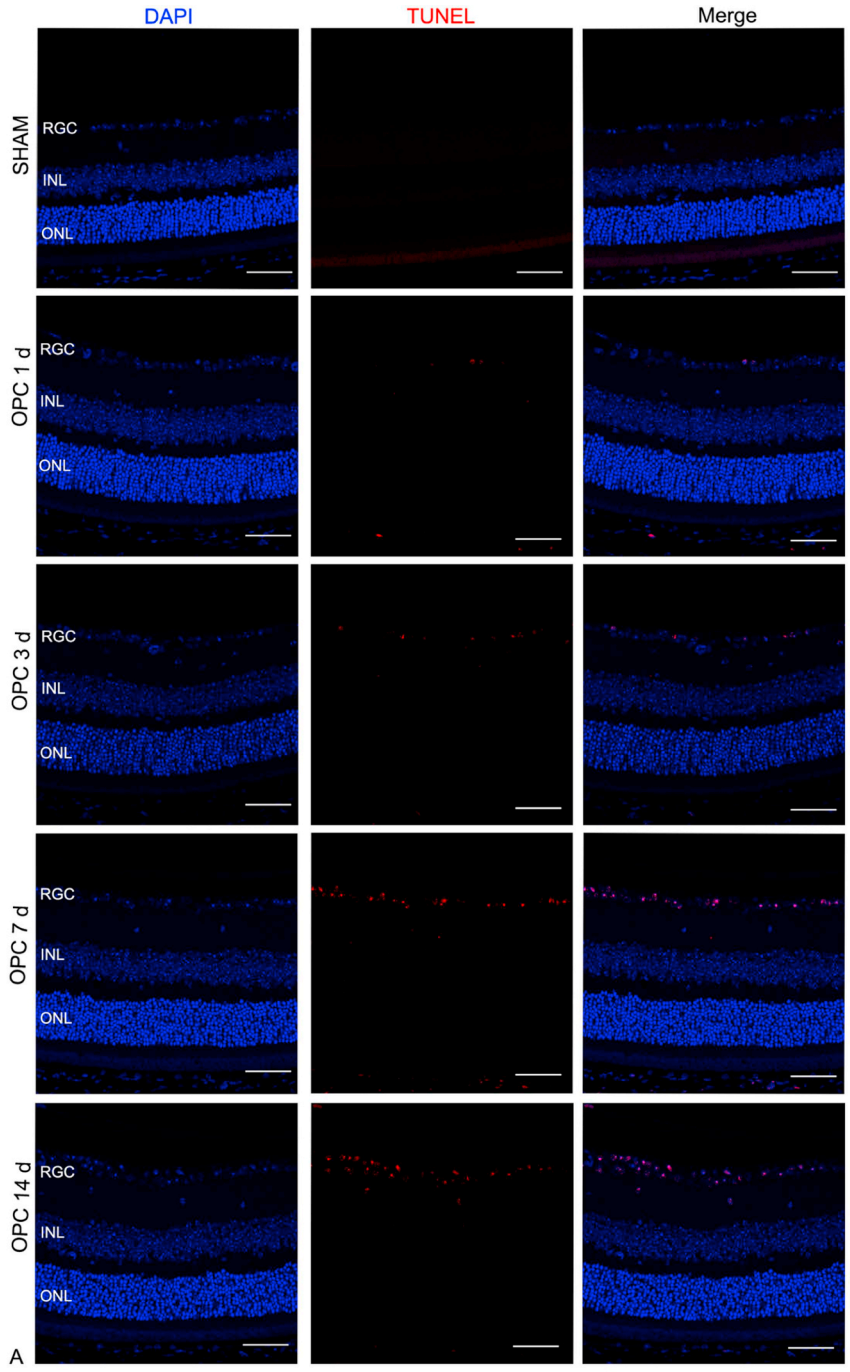


Fig. 4. In the mice with an OPC injury housed in the sterile environment, the mRNA levels of the inflammasome-associated factor pro- IL-1β and the level of the mature IL-1β protein peaked on postoperative day 7, whereas the levels of the HMGB1 and pro-IL-18 mRNAs peaked on postoperative day 14. A: The levels of the pro-IL-1β mRNA in mouse retinas from the surgery and the sham surgery groups were analyzed at different time points after OPC injury in the sterile environment using qPCR; B: The levels of the mature IL-1β protein in mouse retinas from the surgery and the sham surgery groups were analyzed at different time points after OPC injury in the sterile environment using Western blotting. C: The levels of the HMGB1 mRNAs in mouse retinas from the surgery and the sham surgery groups were analyzed at different time points after OPC injury in the sterile environment using qPCR. D: The levels of the pro-IL-18 mRNA in mouse retinas from the surgery and the sham surgery groups were analyzed at different time points after OPC injury in the sterile environment using qPCR. The qPCR data were analyzed using two-way repeated-measures ANOVA; Western blot data were analyzed using one-way ANOVA. All results are presented as the mean ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

levels of all inflammasomes on postoperative day 7 in the mouse OPC injury model between sterile and nonsterile conditions and showed that the relative levels of the Nlrp2, AIM2, and Naip/Nlrc4 inflammasome mRNA were significantly different between mice that received OPC injuries under nonsterile conditions compared to the sham surgery group. However, the levels of these transcripts in mice housed under sterile conditions were not significantly different from the sham surgery group. Based on these results, the expression of these transcripts is closely associated with secondary infection post-operation, and is likely regulated by canonical pathogen clearance response mechanisms. At the same time, the levels of the Nlrp1b and Nlrp3 inflammasome mRNAs changed significantly in mice maintained under both sterile and nonsterile conditions compared with the sham surgery group, indicating that both genes play important roles in both infectious and noninfectious inflammatory responses after OPC injury. The function of Nlrp3 has been confirmed in the sterile inflammation of optic nerve crush in previous reports [10], but the role of Nlrp1 in traumatic optic neuropathy remains unclear. The levels of these two transcripts increased to different extents and at different time points (postoperative days 1, 3, and 7) in mice maintained in the sterile environment. The levels of these two transcripts peaked at different times, indicating that they might have different and important functions at different stages of sterile inflammation.

Sterile inflammation caused by the inflammasome Nlrp1b and Nlrp3 might be associated with the reduction in the number of retinal ganglion cells after optic nerve injury.

Apoptosis is traditionally considered the major pathway responsible for retinal ganglion cells death in traumatic optic neuropathy. However, only some dead ganglion cells express the key apoptosis enzyme caspase-3 [16], suggesting that retinal ganglion cells death in traumatic optic neuropathy might also occur through other mechanisms. Our study confirmed that RIPK3 expression was significantly increased 1–3 d after OPC injury, indicating that necroptosis might be the major pathway of retinal cell death during the acute stage. The mRNA levels of the key apoptosis enzyme caspase-3 and the key pyroptosis enzyme caspase-1 were both increased from postoperative days 3–14, which represent the subacute and chronic stages, respectively. The fold change in the level of the caspase-3 mRNA was less than the value for caspase-1 and peaked on postoperative day 3. The fold changes in the levels of the caspase-1 mRNA and protein were larger and peaked on postoperative day 7. Furthermore, TUNEL staining also showed that this period was the start of the peak stage of retinal ganglion cells death, which reached approximately 60%. Immunohistochemistry revealed high caspase-1 expression in retinal ganglion cells at this time point, and previous studies also reported caspase-1 staining in the retina of the mouse eyeball blast injury model [11], indicating that apoptosis and pyroptosis both occurred during the pathological process at the subacute and chronic stages of OPC injury, and pyroptosis might play the predominant role at the peak stage of cell death. The inflammasome-related Nlrp1b and Nlrp3 mRNAs were both expressed at high levels at the subacute stage; therefore, we speculated that the higher expression of these two transcripts might cause retinal ganglion cells pyroptosis at the subacute stage, 3–7 d after OPC injury. However, further studies are needed to identify which inflammasome protein plays a leading role. The high caspase-1 expression observed on postoperative day 14 might be associated with the cascade reaction of the aforementioned pyroptosis pathway. The levels of the key parthanatos-related enzyme PARP-1 and the key ferroptosis-related gene GPX4 were not increased significantly, suggesting that the two death mechanisms were not initiated in this model [17]. The results obtained from the mice housed under sterile conditions in this study also showed that the relative levels of the Nlrp3 and Nlrp1b mRNAs and mRNAs encoding the



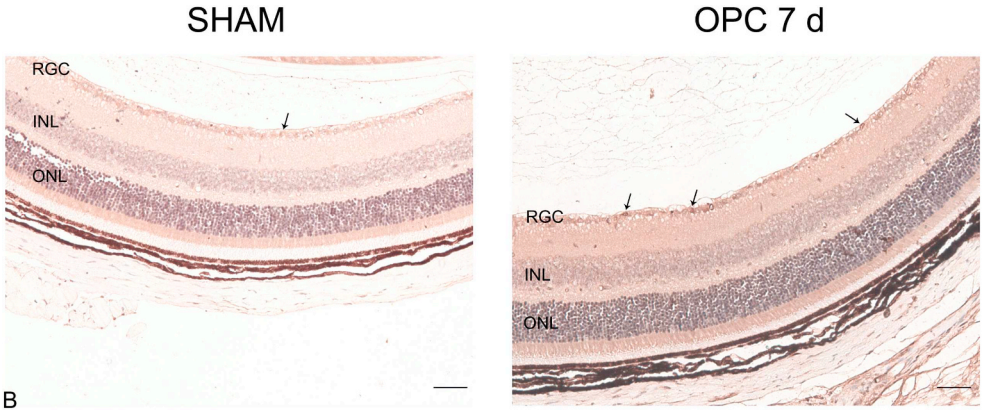


Fig. 5. TUNEL staining and immunohistochemical staining for pro-caspase-1 and cleaved caspase-1 in mouse retinas in which an OPC injury was generated in the sterile environment. A: The retinal ganglion cells of the sham surgery group were TUNEL-negative, and the number of TUNEL-positive retinal ganglion cells increased in the surgery groups throughout the experiment. B: The expression of pro-caspase-1 and cleaved caspase-1 (p20) was detected using immunohistochemical staining on day 7; the expression in the retinas from the mouse OPC injury model and was significantly higher than that in the sham surgery group. The scale bar represents 50 μm.

inflammasome-associated factors pro-IL-1β, pro-IL-18, and HMGB1 were all increased to different extents at different time points after surgery, resulting in the simultaneous development of acute and chronic inflammation in the retina after OPC injury. The relative levels of the IL-1β mRNA and protein were substantially increased on postoperative day 1, which might be associated with the activation of other pathways, such as caspase-8 [18]. The increase in the relative levels of the HMGB1 mRNA observed on postoperative day 14 might be associated with cell death. IL-1β, IL-18 [19], and HMGB1 [12] aggravate sterile inflammatory responses in subjects with traumatic brain injury. Therefore, we speculated that many types of cell death function together in ganglion cells during closed optic neuropathy. By inducing the secretion of relevant factors at the acute and subacute stages, Nlrp3 and Nlrp1b induce excessive inflammation and pyroptosis in tissues, subsequently reducing the number of ganglion cells. Further electrophysiological and knockout animal experiments may be able to help clarify the kinetics of ganglion cell death during this process, and better resolve the contributions of distinct inflammasome components. After all, previous studies using electrophysiology have shown that RGCs may continue to perish up through two weeks following OPC injury, and that these effects may be dependent upon immune cell infiltration and TNF signaling [20,21].

In summary, different cell death pathways were activated at different stages following OPC injury. Cell death was primarily mediated by necroptosis during the acute stage after OPC injury, apoptosis and pyroptosis both occurred during the subacute and chronic stages, and pyroptosis might play a leading role during the subacute cell death stage. Moreover, Nlrp3 and Nlrp1b aggravated sterile inflammation in the retina and its surrounding tissues during the acute and subacute stages to initiate ganglion cell pyroptosis and a vicious cycle resulting in the gradual reduction of ganglion cells. Therefore, at different stages after injury, the activation of different inflammasomes should be targeted to aid in inhibit inflammation, reduce the secondary death of ganglion cells, and effectively protect the optic nerve. Future multi-omics profiling in this animal model may be able to better clarify further the spatiotemporal kinetics of inflammasome activation during OPC injury and identify the stimuli that drives secondary immune activation at these different time points.

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