Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2023**; 28(2): 27
https://doi.org/10.31083/j.fbl2802027

Original Research

Up-Regulation of NLRP3 in the Sclera Correlates with Myopia Progression in a Form-Deprivation Myopia Mouse Model

Zhengyu Chen[1,2], Kang Xiao[1,2], Qin Long[1,2,*]

[1]Department of Ophthalmology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, 100730 Beijing, China
[2]Key Laboratory of Ocular Fundus Diseases, Chinese Academy of Medical Sciences, 100730 Beijing, China
*Correspondence: longqinbj@hotmail.com (Qin Long)
Academic Editor: Josef Jampílek
Submitted: 25 September 2022 Revised: 9 December 2022 Accepted: 13 December 2022 Published: 8 February 2023

Abstract

Background: NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) is a common inflammatory factor that induces inflammation by increasing the expression of related cytokines. Although the NLRP3 inflammasome has been implicated in many ophthalmic diseases, its role in myopia is largely unknown. The aim of this study was to explore the relationship between myopia progression and the NLRP3 pathway. **Methods**: A form-deprivation myopia (FDM) mouse model was used. Different degrees of myopic shift were achieved via monocular form deprivation with 0-, 2-, and 4-week covering, and by 4-week covering followed by 1-week uncovering (the blank, FDM2, FDM4, and FDM5 groups, respectively) in both wild-type and NLRP3 (-/-) C57BL/6J mice. Axial length and refractive power were measured to assess the specific degree of myopic shift. The protein levels of NLRP3 and of related cytokines in the sclera were evaluated by Western blotting and immunohistochemistry. Collagen I and matrix metalloproteinase-2 (MMP-2), which affect extracellular matrix (ECM) remodeling of the sclera, were also examined to clarify the possible underlying mechanism. **Results**: In wild-type mice, the FDM4 group had the most significant myopic shift. Both the increase in refractive power and the elongation in axial length were significantly different between the experimental and control eyes in the FDM2 group. The protein levels of NLRP3, caspase-1, IL-1β, and IL-18 were significantly up-regulated in the FDM4 group compared to the other groups. The myopic shift was reversed and there was less up-regulation of cytokines in the FDM5 group compared to the FDM4 group. MMP-2 expression showed similar trends to NLRP3, while collagen I expression was inversely correlated. Similar results were found in NLRP3 -/- mice, although there was less myopic shift and less obvious changes in cytokine expression in the treatment groups as compared to the wild-type mice. In the blank group, no significant differences were found in refraction and axial length between wild-type mice and NLRP3 -/- mice of the same age. **Conclusions**: NLRP3 activation in the sclera could be involved in myopia progression in the FDM mouse model. Activation of the NLRP3 pathway up-regulated MMP-2 expression, which in turn affected collagen I and caused scleral ECM remodeling, eventually affecting myopic shift.

Keywords: NLRP-3; myopia; inflammation

1. Introduction

Myopia is a highly prevalent eye disease worldwide [1]. An epidemiological survey found that in urbanized East Asia and North America, more than half the children who complete their high school education are myopic [2]. A recent meta-analysis has suggested that close to half of the world's population may be myopic by 2050, with as many as 10% being highly myopic [3]. Low to moderate degrees of myopia make daily life inconvenient due to blurred distance vision. This is usually corrected with spectacles or contact lenses, or by refractive surgery. In some cases, high degree myopia is also correctable using these optical approaches, although there is a significant increase in the incidence of pathological myopia, myopic maculopathy, chorioretinal degeneration, retinal degeneration and detachment [4]. However, permanent vision loss can occur in these situations. The prevention and treatment of myopia, and particularly high degree myopia, has therefore become an important international public health priority [1].

In the vast majority of myopic cases (>95%), the refractive error develops because of excessive axial size resulting from accelerated postnatal eye growth, rather than because of changes in corneal or lens power [5]. Strong evidence from clinical and experimental studies indicates that the sclera plays an important role in determining the size and refractive status of the eye [6,7]. During myopia development, researchers have found that the scleral extracellular matrix (ECM) undergoes remodeling, with decreased stiffness and increased extensibility. This in turn causes the sclera to be less able to withstand normal intraocular pressure, resulting in further posterior expansion and extension, and finally elongation of the ocular axis [8–10]. The most important enzymes involved in this process are matrix metalloproteinases (MMPs), which degrade ECM proteins such as collagen I [11,12].

Previous studies have shown that chronic inflam-

mation is involved in myopia progression [13,14]. The population-based cohort study showed that patients with autoimmune diseases such as type 1 diabetes mellitus, systemic lupus erytheomatosus, uveitis, and allergic conjunctivitis have a higher risk of myopia compared to patients without autoimmune disease. In animal models of myopia, inflammatory markers such as c-Fos, nuclear factor kappa B (NFκB), interleukin (IL)-6 and tumor necrosis factor (TNF)-α are up-regulated. Moreover, IL-6 and TNF-α enhanced the development of myopia, while the immuno-suppressive agent cyclosporine A (CSA) slowed myopia progression. Inflammation is a protective response to pathogen or to damage-associated stimulation. The nucleotide-binding oligomerization domain, leucine-rich repeat–containing receptors (NLRs) are now recognized to be key sensors of pathogens and danger signals. NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) is an intracellular sensor that can detect a broad range of microbial motifs, endogenous danger signals, and environmental irritants. In response to such signals, NLRP3 inflammasomes are formed and activated [15,16]. NLRP3 binds to pro-caspase-1 to form activated NLRP3 inflammasomes, before it shear-activates and releases IL-1β and IL-18 into the extracellular compartment to generate an inflammatory reaction [17]. NLRP3 inflammasomes are mainly found in macrophages, of which a small number are located in the sclera. Previous studies have shown that IL-1β and IL-18 have regulatory effects on MMPs in some tissues, but their effects in the sclera remain unclear [18,19]. Thus, it can be hypothesised that the NLRP-3 pathway affects the progression of myopia by targetting the sclera. The aim of the present study was therefore to investigate the relationship between myopia progression and the NLRP3 inflammasome in the sclera using a form-deprivation myopia (FDM) mouse model.

2. Materials and Methods

2.1 Animals

Healthy 3-week-old male wild-type C57BL/6J mice (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) and NLRP3 (-/-) C57BL/6J mice (ViewSolid Biotech Co., Beijing, China) of the same age and with body weights of 12–14 g were used in this study. Mouse genotypes were confirmed by DNA sequencing technology. Both types of mice were randomly divided into four groups, comprising three FDM groups (12 mice in each group) and a blank group that was comprised of 3 subgroups (6 mice in each subgroup). The FDM mouse model involved covering the right eye with latex balloons for 2 weeks (FDM2 group), 4 weeks (FDM4 group), or 4 weeks followed by 1-week uncovered (FDM5 group). The balloons were also used to make collars that prevented the mice from using their paws to remove the balloon covering their eye. The mice in the blank group did not receive any treatment and were examined when they were 5, 7 and 8 weeks old, thus corresponding to the ages of mice in the FDM2, FDM4 and FDM5 groups, respectively. All mice were exposed to daily periods of 12 hours of natural light and 12 hours of dark. Water, vegetables, and vitamins were provided ad libitum. Animal treatment adhered to the Association for Research in Vision and Ophthalmology's Statement on the Use of Animals in Ophthalmic and Vision Research. The animal experiments were conducted under pathogen-free conditions in line with the institutional animal care protocols approved by the Peking Union Medical College Hospital (XHDW-2022-52).

2.2 Measurements of Refractive Power and Axial Length

Mice were anesthetized with an intraperitoneal injection of 1% pentobarbital sodium and the pupils were then rapidly dilated with compound tropicamide. The refraction of both eyes was measured in a dark room using a banded optometric apparatus (Suzhou Sixty Six Vision Technology Co., Ltd., Suzhou, China; Model YZ24) in the direction of the visual axis. After the mice were sacrificed, their eyes were removed with a forceps and stripped of as much peripheral connective tissue as possible so that the axis of the eye was oriented in the same direction as the micrometer (Dongguan Sanqiang Hardware Machinery Co., Model 211-101). Readings were taken just as the spiral micrometer touched the corneal surface, which determined the axial length (AL). Three measurements were made, and the average value was rounded to the nearest 0.001 mm. All measurements were performed and recorded by experienced personnel.

2.3 Immunohistochemistry

The removed eye samples were fixed in 4% formaldehyde solution at 4 ℃ and then cut into sections, blocked, and incubated with primary antibodies for observation under a fluorescent microscope. For the negative control, normal serum was used instead of the primary antibody. The evaluation of fluorescence was carried out in double-blind fashion. The primary antibodies were specific for NLRP3 (1:200 dilution; #15101, cell signaling technology, USA), IL-1β (1:200; #12242, cell signaling technology, USA), IL-18 (1:200; ab207323, abcam, UK), caspase-1 (1:200; #24232, cell signaling technology, USA), MMP-2 (1:200; ab86607, abcam, UK), and collagen-1 (1:200; ab88147, abcam, UK). BOND polymer refined detection (DS9800, Leica, German) was used. The immunoreactive score (IRS) was determined using a 13-point scale ranging from 0 to 12 and was obtained by multiplying the percentage of positive cells (PP: 0 = no positive cells; 1 = $<$25% positive cells; 2 = 25%–50% positive cells; 3 = 51%–75% positive cells; 4 = $>$75% positive cells) by the predominant degree of staining (SI: 0 = negative; 1 = weak; 2 = moderate; 3 = strong).

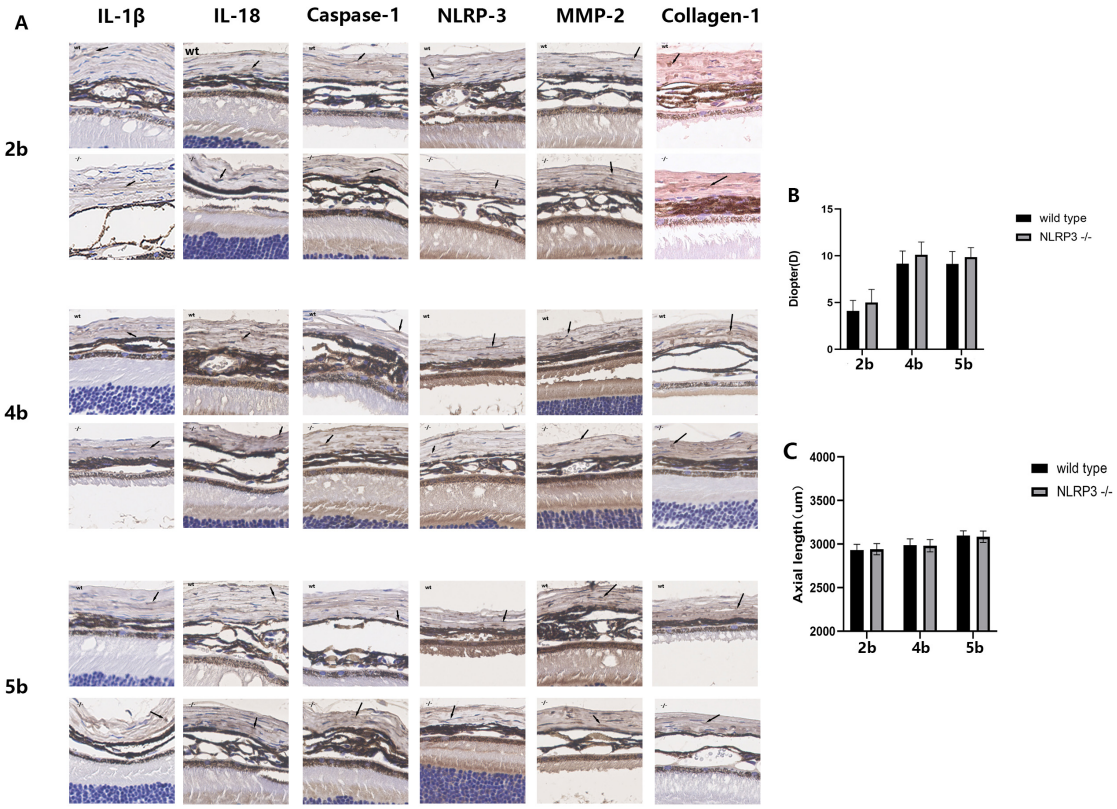

Fig. 1. Refractive status and related cytokine expression in the blank group of wild-type mice and NLRP3-/- mice. 2b, 4b, 5b represent the eyes from the blank group at the same age as the FDM2, FDM4 and FDM5 groups, respectively. (A) Immunohistochemical images of sclera. Each image corresponds to an actual size of 100 μm $\times$ 100 μm. The arrow points to the portion of tissue stained by that antibody. There were no significant differences in refraction (B) or axial length (C) between NLRP3 -/- and wild-type mice in the blank group at the same age.

2.4 Western Blotting

The sclera was snap-frozen in liquid nitrogen, ground into a powder and then placed in lysis solution for 60 minutes. The scleral lysates were prepared in SDS lysis buffer containing a mixture of protease and phosphatase inhibitors (Pierce, Rockford, IL). The proteins were quantified using a Bradford protein assay and 40 μg samples of total protein were loaded onto sodium dodecyl sulfate–polyacrylamide gels. Following separation by electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). These were incubated overnight at 4 °C with the same primary antibodies as those used for immunohistochemistry. Goat anti-rabbit (ab6721, abcam) or anti-mouse (ab6789, abcam) horseradish peroxidase-conjugated secondary antibody was used to detect target proteins with a western blotting kit (12957s, Cell Signaling Technology), followed by scanning with the Odyssey Fc System (LI-COR, USA). Image J 1.8 software (LOCI, University of Wisconsin, Madison, WI, USA) was used for grayscale analysis, with the relative gray level obtained by dividing the target band with β-actin to provide a semi-quantitative measure of protein expression level.

2.5 Statistical Analysis

SPSS 22.0 (IBM Corp., Chicago, IL, USA) statistical software was used for statistical analysis. Measurement data in this study were tested for normality using the Shapiro-Wilk test and expressed as the mean $\pm$ standard deviation. For the comparison of groups, differences were assessed using parametric tests such as the *t*-test (for normally distributed data), the Mann-Whitney U test for non-parametric data, or the Wilcoxon matched pairs signed rank test. p-values < 0.05 were considered statistically significant.

3. Results

3.1 NLRP3 -/- Mice Achieved a Similar Refractive Status during Natural Development as Wild-Type Mice

To determine whether the myopic shift in wild-type and NLRP3 -/- mice was comparable in the subsequent study, we first compared the refractive status of the blank group in both wild-type and NLRP3 -/- mice. No significant differences were found in axial length (AL) or refraction power between the two mouse types at the same age (Fig. 1B,C). Immunohistochemical staining showed that

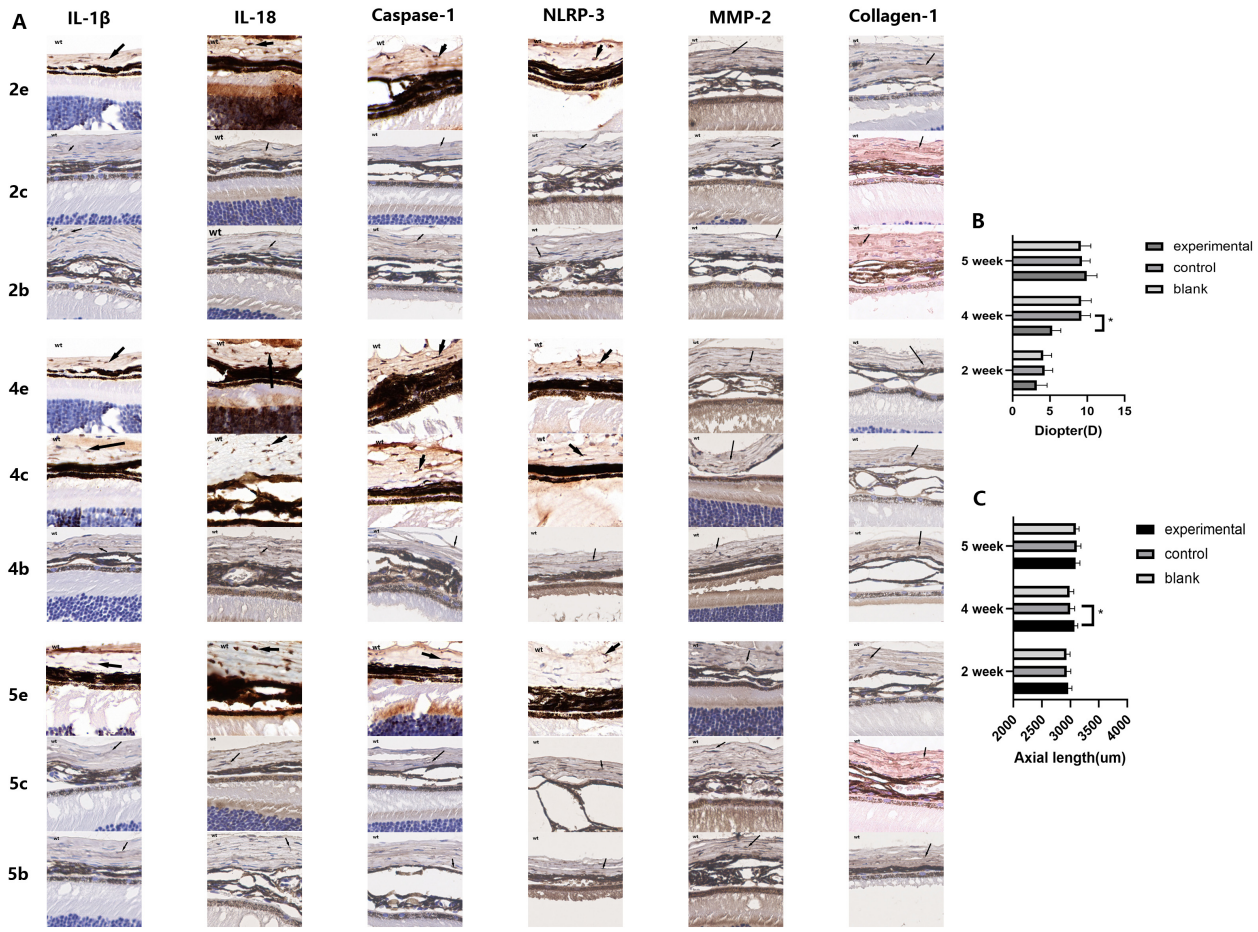

Fig. 2. The expression of inflammatory cytokines related to the NLRP3 pathway and of scleral tissue remodeling proteins increased over time in experimental eyes, and decreased after removal of the eye mask. Form deprivation promoted myopic shift in wild-type C57/BL6J mice. 2c represents the control eyes in the FDM2 group, 2e represents the experimental eyes in the FDM2 group, 2b represents the eyes in the blank group of mice with the same age as FDM2, and so on for FDM4 and FDM5. (A) Immunohistochemical images of sclera. Scleral tissues were obtained from wild-type mice. Each image corresponds to an actual size of 100 μm $\times$ 100 μm, with the arrow pointing to the portion of tissue stained with the antibody. Quantitative analysis of diopter (B) and axial length (C) from the wild-type mice used in the experiments (n = 12). * represents a statistically significant difference (group A vs.group B, $p < 0.05$) between the two groups connected by the solid line.

NLRP3 was expressed at much lower levels in NLRP3 -/- mice compared to wild-type mice. Other cytokines related to the NLRP3 pathway and to the scleral ECM, including IL-1β, IL-18, caspase-1, MMP-2 and collagen-1, also showed differences in expression, but these did not reach statistical significance (IRS in Table 1). Hence, in the absence of ocular masking, there were no differences in refractive status between wild-type and NLRP3 -/- mice.

3.2 The NLRP3 Inflammasome is Activated in the Scleral Tissue of FDM Mice

We next investigated whether changes to the NLRP3 inflammasome in the sclera were associated with myopia development. To do this, the myopic shift, AL elongation, and expression levels of NLRP3 and caspase-1 in the experimental eyes were compared with the control eyes (i.e., the uncovered contralateral eye) in the FDM2 and FDM4 groups of wild-type mice.

In the FDM4 group, significant myopic shift (Fig. 2C; 5.30 $\pm$ 1.16 D vs. 9.20 $\pm$ 1.21D, $p = 0.001$) and AL elongation (Fig. 2B, 3070 $\pm$ 130 D vs. 2945 $\pm$ 115 D, $p = 0.03$) were observed in the experimental eyes compared to control eyes, respectively. A similar trend was seen in the FDM2 group, but the differences in myopic shift (Fig. 2C; 3.25 $\pm$ 1.37 D vs. 4.30 $\pm$ 1.08 D, $p = 0.08$) and AL elongation (Fig. 2B; 2964 $\pm$ 127 D vs. 2832 $\pm$ 193 D, $p = 0.13$) did not reach statistical significance.

Western blotting and immunohistochemistry revealed that the expression levels of NLRP3 and caspase-1 protein were both correlated with the duration of form deprivation. In both the FDM2 and FDM4 groups, the NLRP3 and caspase-1 levels in the sclera of experimental eyes were

Table 1. Immunoreactive score (IRS) in the sclera of wild-type and NLRP3 -/- mice (n = 6).

	2c	2b	2e	4c	4b	4e	5c	5b	5e
	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
IL-1β	1.2 ± 1.0/2.4 ± 1.2	0.9 ± 1.1/1.8 ± 1.3	5.1 ± 1.8/4.1 ± 1.6	2.1 ± 1.1/4.3 ± 1.5	1.8 ± 1.3/3.8 ± 1.7	9.5 ± 2.9/7.3 ± 2.0	2.9 ± 1.6/3.8 ± 1.4	2.6 ± 1.4/3.4 ± 1.6	5.4 ± 1.6/5.0 ± 1.8
IL-18	3.3 ± 1.6/2.2 ± 1.4	2.7 ± 1.4/1.9 ± 1.6	7.3 ± 2.3/4.6 ± 1.6	6.2 ± 2.9/5.6 ± 1.9	6.1 ± 3.1/4.9 ± 1.8	8.8 ± 2.6/8.2 ± 2.2	4.0 ± 2.1/4.2 ± 1.6	4.7 ± 1.9/4.4 ± 1.4	8.0 ± 1.9/5.4 ± 1.7
Caspase-1	1.5 ± 0.9/3.3 ± 1.2	1.8 ± 0.8/2.6 ± 1.5	4.3 ± 1.4/3.7 ± 1.3	1.8 ± 0.8/4.2 ± 1.5	2.0 ± 1.1/4.8 ± 1.8	8.5 ± 1.8/6.7 ± 1.6	2.6 ± 1.4/3.0 ± 1.6	2.3 ± 1.2/3.2 ± 1.4	1.6 ± 0.9/6.1 ± 2.1
NLRP-3	2.4 ± 1.1/0.8 ± 0.6	2.2 ± 1.0/1.1 ± 0.5	5.7 ± 1.4/1.5 ± 0.7	3.0 ± 1.3/1.6 ± 0.8	3.5 ± 1.1/1.3 ± 0.6	7.4 ± 1.9/1.4 ± 0.5	3.1 ± 2.0/1.6 ± 0.8	3.4 ± 1.6/1.1 ± 0.9	2.2 ± 1.2/1.1 ± 0.4
MMP-2	1.1 ± 0.8/3.5 ± 1.6	1.6 ± 1.1/2.7 ± 1.4	7.6 ± 2.2/6.0 ± 1.8	4.8 ± 1.6/2.8 ± 0.8	5.1 ± 1.8/2.1 ± 0.9	9.4 ± 2.4/7.8 ± 2.1	3.0 ± 1.6/3.8 ± 1.8	3.0 ± 1.9/3.4 ± 1.6	3.5 ± 1.4/3.0 ± 1.4
Collagen-1	4.7 ± 2.1/5.7 ± 2.0	5.0 ± 1.8/5.4 ± 2.2	3.5 ± 1.4/5.7 ± 1.4	3.3 ± 1.3/6.4 ± 2.0	4.0 ± 1.9/5.2 ± 1.5	2.1 ± 0.8/4.6 ± 1.5	4.8 ± 1.3/5.5 ± 2.3	4.4 ± 1.6/5.7 ± 1.8	2.8 ± 1.2/6.0 ± 2.3

The values shown in the table are the average IRS from 6 eyes in the same group. Data are expressed as the mean ± SD. 2c represents the control eyes (the uncovered contralateral eye in the FDM group) in the FDM2 group, 2e represents the experimental eyes in the FDM2 group, 2b represents the eyes in the blank group with the same age as the FDM2 group, and so on for FDM4 and FDM5. '+' represents data from wild-type mice, while '-' is data from NLRP3 -/- mice.

higher than in the control eyes (Fig. 3A–C, and Fig. 2A). NLRP3 and caspase-1 expression in the experimental eyes of the FDM4 group were higher than in the control eyes of the same group (Fig. 3B,C, $p = 0.006$), and also higher than in the experimental eyes of the FDM2 group (Fig. 3B,C, $p = 0.032$). This was consistent with the results observed for myopic shift and AL elongation. Similar results were also obtained using the immunohistochemical semi-quantitative method for comparison (Table 1). These findings suggest that myopic progression and activation of the NLRP3 inflammasome pathway occur in the FDM mouse model.

3.3 Suppression of the NLRP3 Pathway Following Removal of the Mask Induced a Hyperopic Shift

The relationship between NLRP3 expression and myopia was further investigated by comparing the AL, myopic shift, and NLRP3 expression in the experimental eyes of wild-type mice in the FDM5 group with those from the FDM4 group. One week after removal of the covering, the expression levels of NLRP3 and caspase-1 were significantly lower, as observed by Western blotting (Fig. 3B,C) and immunohistochemistry (Fig. 2A). The myopic shift in the experimental eyes was also significantly reduced (Fig. 2B; 9.90 ± 1.40 vs. 5.30 ± 1.16, $p = 0.001$), but there was no significant difference between the experimental and control eyes (Fig. 2B; 9.90 ± 1.40 vs. 9.27 ± 1.10, $p = 0.18$) in the FDM5 group. In addition, there was no statistical difference in AL between the experimental and control eyes in the FDM5 group (Fig. 2C; 3191 ± 99 vs. 3205 ± 122, $p = 0.67$), whereas the experimental eyes had significantly longer AL in the FDM4 group ($p = 0.03$). Therefore, the decreased NLRP3 and caspase-1 expression levels after removal of the mask resulted in a subsequent hyperopic shift in the experimental eyes. The control eyes in each group were also compared with those in the blank group, but no significant differences were found in the expression levels of each factor, AL or refraction (Fig. 2). Hence, we concluded that the increased expression level of each factor in the experimental eye was confined to that eye and was not due to systemic inflammation.

3.4 NLRP3 Deletion Reduced FDM Development

To clarify the effect of NLRP3 on the development of myopia, we examined FDM using NLRP3 -/- mice and measured the refraction power, AL and expression levels of NLRP3 and caspase-1 in the different experimental groups. The results were compared with those of wild-type mice from the same age group.

Refractive changes in the experimental eyes from the FDM4 group of NLRP3 -/- mice were again significantly different to those of control eyes (Fig. 4B; 7.33 ± 1.16 vs. 9.15 ± 1.24, $p = 0.01$). However, there was no significant difference between the experimental and control eyes in the FDM2 group of NLRP3-/- mice (Fig. 4B; 3.75 ± 1.37 vs. 4.22 ± 1.31, $p = 0.47$). This differed to the result in wild-type mice, where a trend was observed ($p = 0.08$). Cross-group comparison of the same age group revealed that the tendency towards myopia was suppressed in the experimental eyes of NLRP3 -/- mice (FDM4: wild-type vs. NLRP3 -/-: 7.33 ± 1.16 vs. 5.30 ± 1.07, $p = 0.0002$). The change in AL was similar, but the difference was not statistically significant (FDM4: wild-type vs. NLRP3 -/-: 3070 ± 130 vs. 3012 ± 115, $p = 0.26$). The difference in AL between experimental and control eyes in FDM4 group of NLRP3 -/- mice was not significant (Fig. 4C, 3012 ± 115 vs. 2960 ± 133, $p = 0.31$), in contrast to the significant difference observed with wild-type mice ($p = 0.03$).

Furthermore, NLRP3 expression in NLRP3 -/- mice was not statistically different between the various groups (Fig. 5D and Fig. 4A) and relatively low (Fig. 6B Table 1). The expression level for Caspase-1 in the experimental eyes of the FDM4 group was higher than in the other groups of NLRP3 -/- mice (Fig. 5F), but significantly lower than that observed in wild-type mice (Fig. 6A). The myopic shift in the FDM mouse model was suppressed when NLRP3 expression was lower.

3.5 NLRP3 Enhances Myopia via Downstream Cytokines and the Expression of Proteins for Scleral Integrity

To explain the above findings, we measured the expression of IL-18 and IL-1β, and of MMP-2 and collagen 1. These represent factors downstream of the NLRP3 signaling pathway, and factors associated with scleral integrity and myopia progression, respectively. In wild-type mice, the expression levels of IL-1β and MMP-2 in the experimental eyes were higher than in the control eyes in both the FDM2 and FDM4 groups. The expression levels in the experimental eyes of the FDM4 group were also higher than those of the FDM2 group. However, the expression levels in the experimental eyes of the FDM5 group were lower compared to the FDM4 group (Fig. 3D,G and Table 1). The results in NLRP3 -/- mice were identical to those of wild-type mice (Fig. 5B,G and Table 1), consistent with the myopic shift and changes in NLRP3 expression level. These results indicate a positive correlation between the expression levels of IL-1β and MMP-2, and also that increased expression of these two factors promoted the progression of myopia.

The expression of collagen I was completely different to IL-1β and MMP-2. In each group of wild-type mice, collagen I expression was higher in the control eyes than in the experimental eyes (Fig. 3G), while its expression in the experimental eyes of the FDM4 group was lower than in all other groups (Fig. 2A). In NLRP3 -/- mice, collagen I expression was also lowest in the experimental eyes of the FDM4 group, but no statistical differences were observed compared to the other groups (Fig. 5C). Since collagen I is a major component of the scleral ECM, IL-1β and MMP-2 may promote the progression of myopia by down-regulating collagen-1 and subsequent scleral ECM remod-

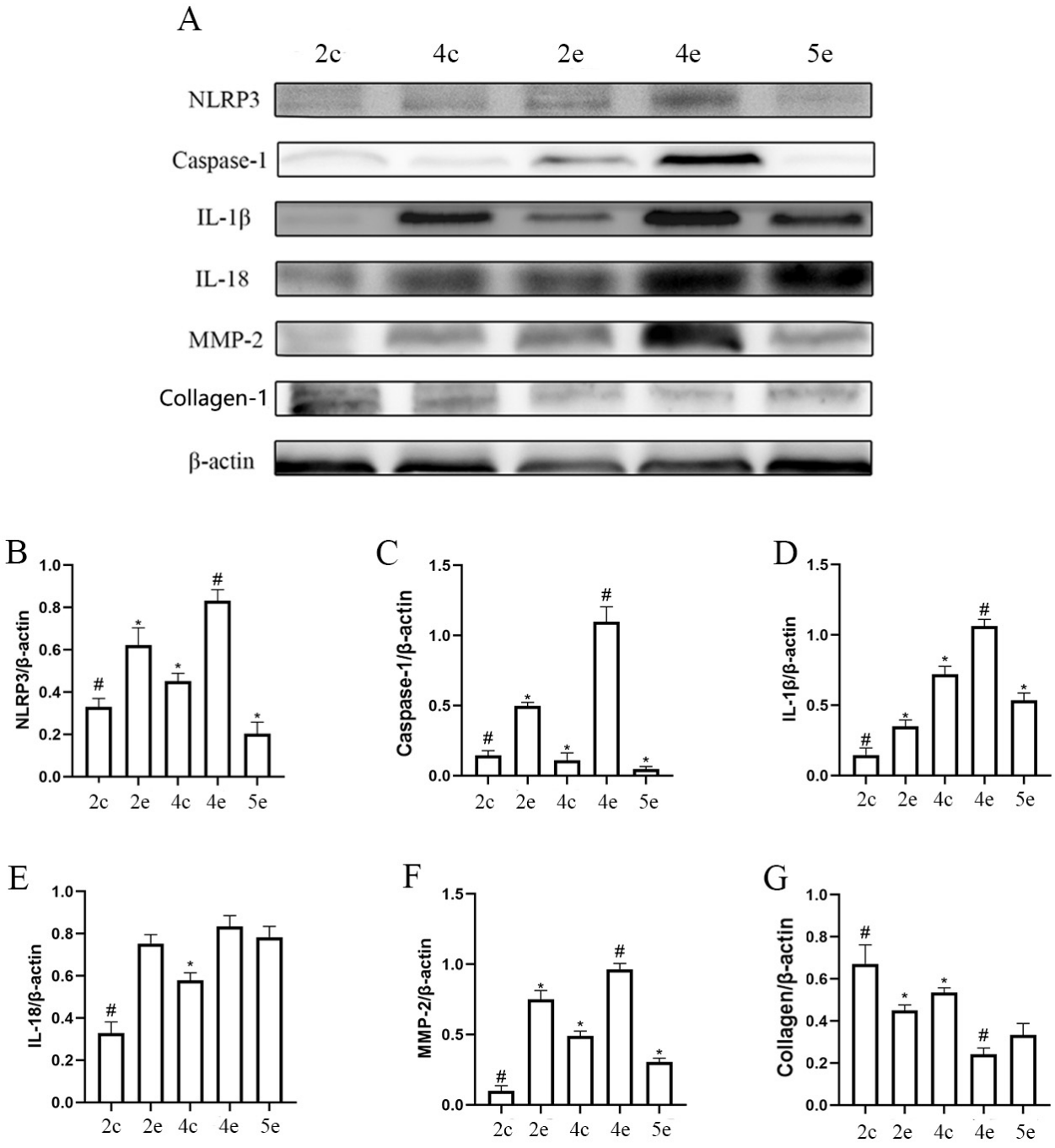

Fig. 3. Form deprivation promoted scleral NLRP3 inflammasome activation in wild-type C57/BL6J mice. 2c represents the control eyes in the FDM2 group, 2e represents the experimental eyes in the FDM2 group, and so on for the FDM4 and FDM5 groups. (A) Representative Western blot results for NLRP3, caspase-1, IL-1β, IL-18, MMP-2 and collagen-1 protein levels. Semi-quantitative analysis of NLRP3 (B), caspase-1 (C), IL-1β (D), IL-18 (E), MMP-2 (F) and collagen-1 (G) expression levels deduced from the Western blot results. Data are expressed as mean $\pm$ SD. (n = 3). # means 'this group vs. 2e group, $p < 0.05$' , and * means 'this group vs. 4e group, $p < 0.05$'.

eling.

In wild-type mice, IL-18 showed high expression in all experimental eyes, with no statistical difference between groups. In contrast, the experimental eyes in the FDM4 group of NLRP3 -/- mice showed a statistically different level of IL-18 expression to all of the other groups (Fig. 5E), although they also showed reduced levels compared to wild-type mice (Fig. 6D). It is possible that the FDM model induces IL-18 activation through pathways other than NLRP3/caspase-1, therefore explaining why masking

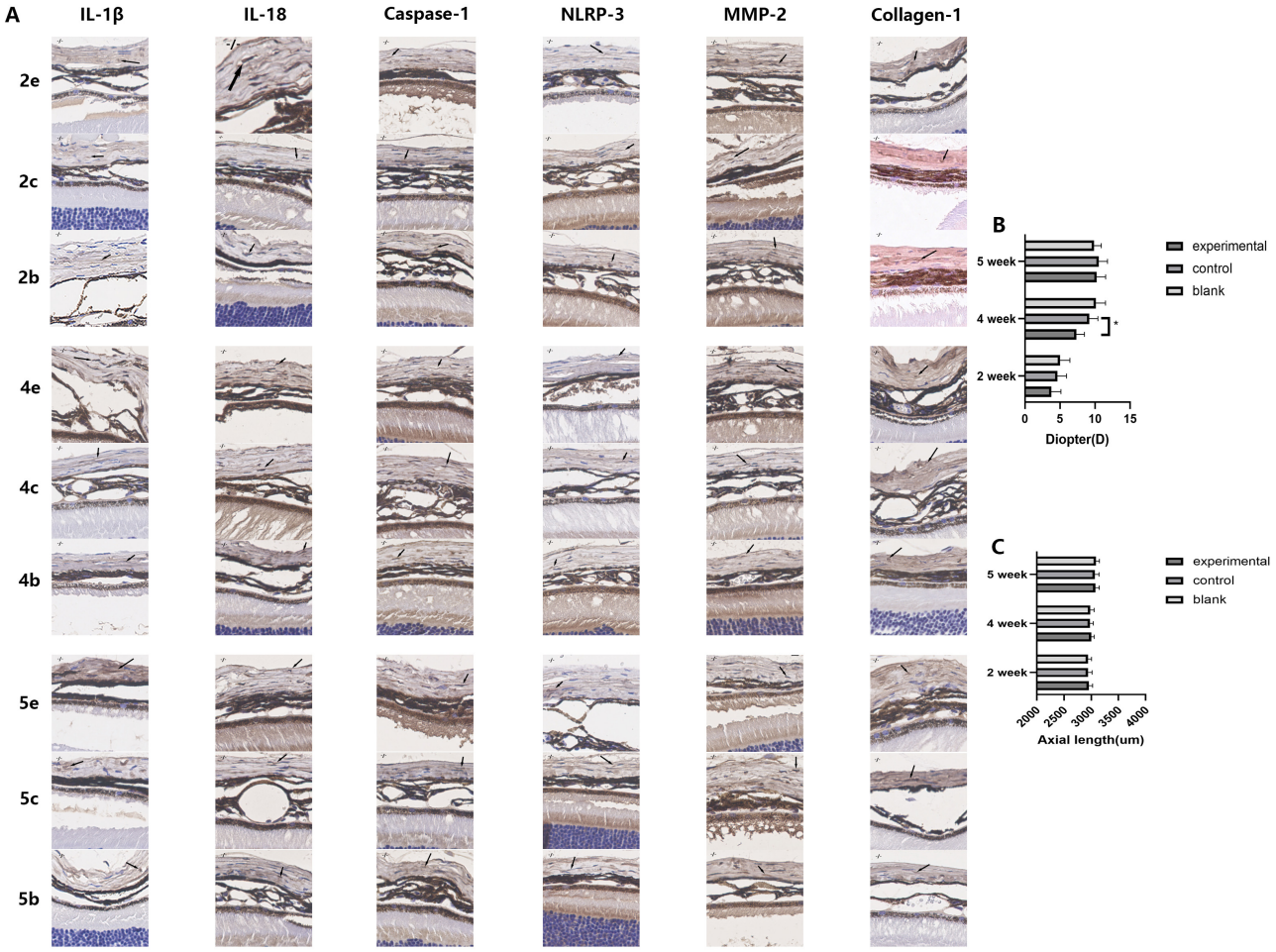

Fig. 4. The expression of inflammatory cytokines related to the NLRP3 pathway and of scleral tissue remodeling proteins increased over time in NLRP3 -/- mice, but this did not reach statistical significance. 2c represents the control eyes in the FDM2 group, 2e represents the experimental eyes in the FDM2 group, 2b represents the eyes in the blank group with the same age as the FDM2 group, and so on for the FDM4 and FDM5 groups. (A) Immunohistochemical images of scleral tissues obtained from NLRP3 -/- mice. Each image corresponds to an actual size of 100 μm $\times$ 100 μm, with the arrow pointing to the area of tissue stained by that antibody. Quantitative analysis of diopter (B) and axial length (C) from the NLRP3 -/- mice used in the experiments (n = 12). * represents a statistical difference (group A vs. group B, $p < 0.05$) between the two groups connected by the solid line.

still increased IL-18 expression in NLRP3 -/- mice.

In the FDM4 group of NLRP3 -/- mice, no statistical differences were observed between the expression of each factor in the eyes of the blank group compared to the eyes of the controls. This demonstrates that the FDM model did not affect the expression level of each factor in the control eyes of NLRP3 -/- mice (Fig. 5B–G).

4. Discussion

Axial elongation is the most important form of change in the progression of myopia [20], and was recapitulated in the FDM mouse model used in the current study. In the FDM4 group, the refraction shift towards myopia and axial elongation were both significantly different in the experimental eyes compared to the control eyes. Moreover, the difference diminished after the mask had been removed for one week. These results confirm a successful establishment of the myopic model and validate the findings of previous studies [21]. There were no significant differences in either refraction or AL between eyes in the blank group and control eyes in the FDM4 group, suggesting that masking one eye did not affect development of the unmasked control eye. Together with the observation of no significant differences in myopic shift and AL elongation between the control eyes in the FDM4 group and the eyes in the blank group, we believe that monocular form deprivation in wild-type mice has no significant effect on the untreated eye in FDM models.

In this study, immunohistochemistry and Western blotting showed that protein expression levels for NLRP3, Caspase-1, IL-18, and MMP-2 were highly consistent with myopic shift and AL elongation in wild-type mice. These

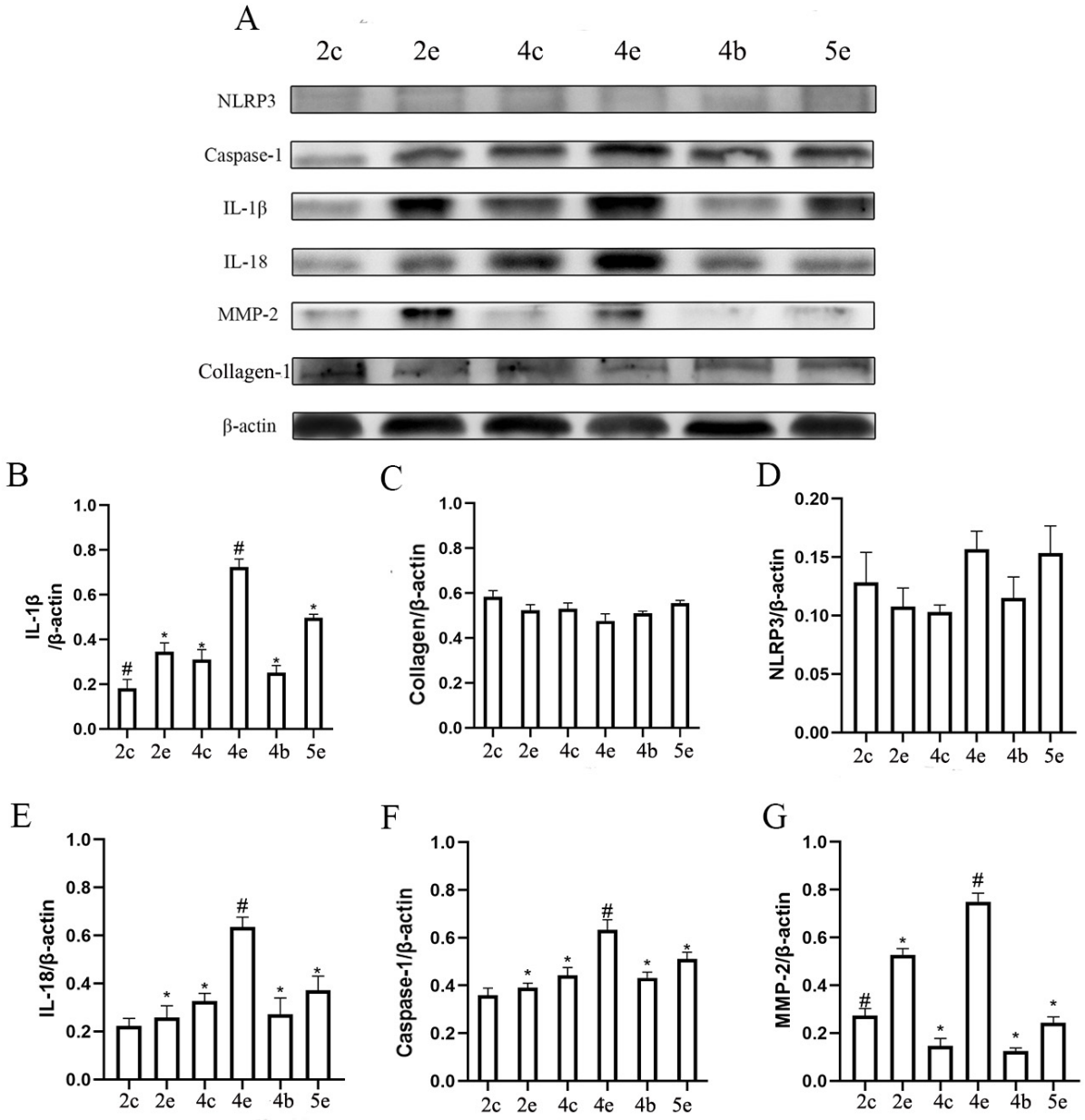

Fig. 5. NLRP3 knockout reduced scleral NLRP3 inflammasome activation in the FDM mouse model. 2c represents the control eyes in the FDM2 group, 2e represents the experimental eyes in the FDM2 group, and so on for the FDM4 and FDM5 groups. (A) Representative Western blot for expression of the NLRP3, caspase-1, IL-1β, IL-18, MMP-2 and collagen-1 proteins. Semi-quantitative analysis for NLRP3 (D), caspase-1 (F), IL-1β (B), IL-18 (E), MMP-2 (G) and collagen-1 (C) expression levels deduced from the Western blot results. Data are expressed as mean $\pm$ SD (n = 3). # means 'this group vs. 2e group, $p < 0.05$' ; * means 'this group vs. 4e group, $p < 0.05$'.

results strongly suggest the NLRP3 pathway is associated with the progression of myopia. Previous studies have shown that NLRP3 is involved in various eye diseases such as keratitis, diabetic retinopathy, dry eye disease and age-related macular degeneration [22–24]. The current study has for the first time revealed an association between the NLRP3 pathway and myopia.

The role of inflammation in myopia progression has been described previously [25,26]. Lin and colleagues reported that expression levels for inflammatory cytokines including c-Fos, nuclear factor κB, IL-6, and tumor necrosis factor-α were up-regulated in the FDM model, and down-regulated by treatment with atropine [13]. Furthermore, these authors found that myopia progression could be slowed by the immunosuppressive agent cyclosporine A. Our findings provide additional evidence for the involvement of an inflammatory response in the pathogenesis of myopia.

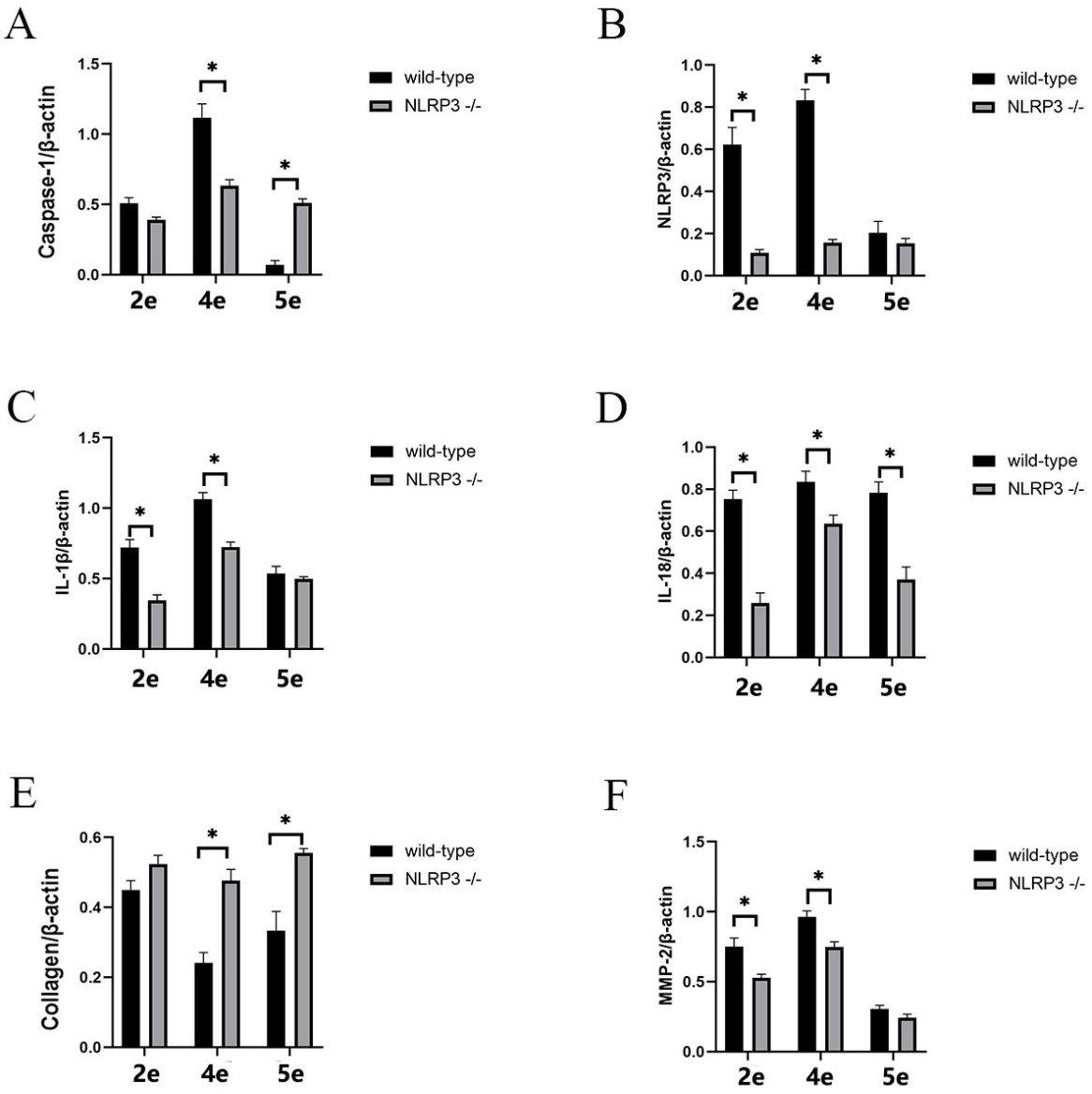

Fig. 6. Deletion of NLRP3 reduces the development of FDM and the expression of inflammatory cytokines related to NLRP3. Comparison of caspase-1 (A), NLRP3 (B), IL-1β (C), IL-18 (D), collagen-1 (E), and MMP-2 (F) expression levels in NLRP3 -/- and wild-type mice of the same age and group using relative grey-scale values. The color shades and ranges detected by a specific antibody in western blot were quantified in image J as grayscale values and then divided by the grayscale value of its corresponding β-actin. 2e represents the experimental eyes in the FDM2 group, and so on for the FDM4 and FDM5 groups.* represents a statistically significant difference (group A vs.group B, $p < 0.05$'.) between the two groups connected by the solid line.

Previous studies have demonstrated that MMP-2 can induce scleral ECM remodeling, thereby playing a key role in myopia development. IL-18 and IL-1β can up-regulate MMP-2 in many types of cells [18,27,28]. Zhao *et al*. [29] reported that scleral monocyte-derived macrophages contribute to the development of myopia by increasing MMP-2 expression in FDM mice. In the present study, protein expression levels for NLRP3, Caspase-1, IL-1β, and MMP-2 were positively correlated with myopic shift and AL elongation in wild-type mice. In NLRP3 -/- mice, the expression of NLRP3 was suppressed, the expression levels for Caspase-1, IL-1β, and MMP-2 were also lower to varying degrees, and myopic shift and AL elongation were reduced. In addition, collagen I expression was inversely correlated with NLRP3 expression. Combined with the findings of previous studies, we propose the following pathway in the FDM model: masking the eye causes NLRP3 expression to increase; NLRP3 binds to pro-Caspase-1 to form the NLRP3 inflammasome and activate Caspase-1; Caspase-1 converts pro-IL-1β to IL-1β, which induces increased expression of MMP-2; MMP-2 then down-regulates collagen I expression, which ultimately leads to remodeling of the

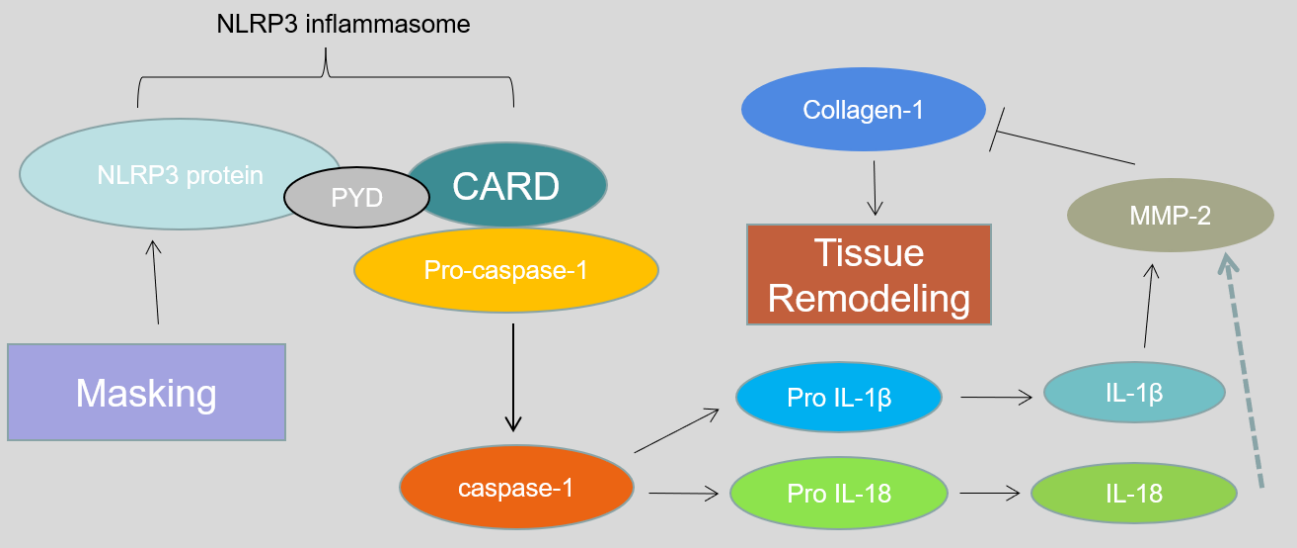

Fig. 7. Possible mechanism explaining how the up-regulation of NLRP3 induces a myopic shift.

scleral ECM and progression of myopia (Fig. 7).

NLRP3 -/- mice experienced a significant myopic shift in the form-deprivation eyes compared with control eyes, although to a much lesser extent than in wild-type mice. Similiar trends were observed for axial elongation. A possible explanation could be that NLRP3 was only partially suppressed in the knockout mice used in this study, and the residual expression of NLRP3 could still play a role in the development of myopia. Another explanation is that myopia is a multifactorial disease, and hence the progression of myopia is only partly determined by NLRP3 activation. The FDM model could also undergo myopia progression through other pathways.

The situation with IL-18 was somewhat complicated by the fact that its generally high expression in the experimental eyes of wild-type mice prevented us from determining whether it was also involved in the aforementioned mechanism of myopia progression. However, the expression level of IL-18 in NLRP3 -/- mice was consistent with a myopic shift. This suggests there are other pathways in the FDM model besides NLRP3 that can increase the IL-18 expression level and thus be associated with myopia progression.

Despite a plethora of research on myopia, the molecular/cellular mechanisms that underlie its pathology are still not well understood, thereby slowing the search for more effective pharmacological controls. So far, the anticholinergic blocking agent atropine has been the most effective and is used by clinicians in off-label ways for myopia control. However, there are concerns regarding potential long-term ocular or systemic side-effects of atropine usage. NLRP3 is a common inflammatory factor involved in the development and progression of many eye diseases. Inhibitors of NLRP3 have proven effective in the treatment of these diseases [30–32]. The present study demonstrated the involvement of NLRP3 in the progression of myopia in the FDM model. Moreover, the suppression of NLRP3 expression reduced the extent of myopic shift, suggesting that NLRP3 inhibitors could potentially be used to prevent or slow the development of myopia.

A limitation of this study was that only protein expression levels were assessed, with no analysis at the transcriptional level using methods such as RT-PCR. In addition, Western blotting and immunohistochemistry are semi-quantitative descriptions and somewhat inadequate for precise numerical evaluation.

5. Conclusions

In summary, this is the first report to our knowledge that NLRP3 could be involved in myopic shift in the FDM mouse model. The underlying mechanism might be that NLRP3 promotes the activation of IL-1β, thereby inducing MMP-2 expression, which in turn affects collagen I and causes scleral ECM remodeling, thus eventually played a role in myopic shift. These results provide a basis for future investigations into the possible use of NLRP3 inhibitors for the treatment of myopia.

Availability of Data and Materials

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

Author Contributions

ZC designed the research study. ZC and KX performed the research. QL provided help and advice on the research design and paper writing. ZC analyzed the data. ZC wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Animal treatment adhered to the Association for Research in Vision and Ophthalmology's Statement for the Use of Animals in Ophthalmic and Vision Research, and the animal experiments were conducted under pathogen-free conditions in line with the institutional animal care protocols approved by the Peking Union Medical College Hospital (XHDW-2022-52).

Acknowledgment

The authors of this article would like to thank Yongfeng Lv and Lu Sun for their guidance on optometry techniques.

Funding

This research was funded by National Natural Science Foundation of China (Number 81870685) and the Natural Science Foundation of Beijing Municipality (Number 7172173).

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Morgan IG, Ohno-Matsui K, Saw S. Myopia. Lancet (London, England). 2012; 379: 1739–1748.

[2] Wu P, Huang H, Yu H, Fang P, Chen C. Epidemiology of Myopia. Asia-Pacific Journal of Ophthalmology. 2016; 5: 386–393.

[3] Holden BA, Fricke TR, Wilson DA, Jong M, Naidoo KS, Sankaridurg P, *et al*. Global Prevalence of Myopia and High Myopia and Temporal Trends from 2000 through 2050. Ophthalmology. 2016; 123: 1036–1042.

[4] Spaide RF, Ohno-Matsui K, Yannuzzi LA. Pathologic Myopia. Springer: New York. 2014.

[5] Zadnik K. The Glenn A. Fry Award Lecture (1995). Myopia development in childhood. Optometry and Vision Science: Official Publication of the American Academy of Optometry. 1997; 74: 603–608.

[6] McBrien NA. Regulation of scleral metabolism in myopia and the role of transforming growth factor-beta. Experimental Eye Research. 2013; 114: 128–140.

[7] Rada JAS, Shelton S, Norton TT. The sclera and myopia. Experimental Eye Research. 2006; 82: 185–200.

[8] Marzani D, Wallman J. Growth of the two layers of the chick sclera is modulated reciprocally by visual conditions. Investigative Ophthalmology & Visual Science. 1997; 38: 1726–1739.

[9] Phillips JR, Khalaj M, McBrien NA. Induced myopia associated with increased scleral creep in chick and tree shrew eyes. Investigative Ophthalmology & Visual Science. 2000; 41: 2028–2034.

[10] Guo L, Frost MR, He L, Siegwart JT, Norton TT. Gene expression signatures in tree shrew sclera in response to three myopiagenic conditions. Investigative Ophthalmology & Visual Science. 2013; 54: 6806–6819.

[11] Guggenheim JA, McBrien NA. Form-deprivation myopia induces activation of scleral matrix metalloproteinase-2 in tree shrew. Investigative Ophthalmology & Visual Science. 1996; 37: 1380–1395.

[12] Yue Y, Hsiao Y, Zhou J. Association between MMP/TIMP Levels in the Aqueous Humor and Plasma with Axial Lengths in Myopia Patients. BioMed Research International. 2020; 2020: 2961742.

[13] Lin H, Wei C, Chang C, Chen T, Hsu Y, Hsieh Y, *et al*. Role of Chronic Inflammation in Myopia Progression: Clinical Evidence and Experimental Validation. EBioMedicine. 2016; 10: 269–281.

[14] Wei C, Kung Y, Chen CS, Chang C, Lin C, Tien P, *et al*. Allergic Conjunctivitis-induced Retinal Inflammation Promotes Myopia Progression. EBioMedicine. 2018; 28: 274–286.

[15] Swanson KV, Deng M, Ting JP. The NLRP3 inflammasome: molecular activation and regulation to therapeutics. Nature Reviews. Immunology. 2019; 19: 477–489.

[16] Strowig T, Henao-Mejia J, Elinav E, Flavell R. Inflammasomes in health and disease. Nature. 2012; 481: 278–286.

[17] Shalhoub J, Falck-Hansen MA, Davies AH, Monaco C. Innate immunity and monocyte-macrophage activation in atherosclerosis. Journal of Inflammation (London, England). 2011; 8: 9.

[18] Abraham M, Shapiro S, Lahat N, Miller A. The role of IL-18 and IL-12 in the modulation of matrix metalloproteinases and their tissue inhibitors in monocytic cells. International Immunology. 2002; 14: 1449–1457.

[19] Mountain DJH, Singh M, Menon B, Singh K. Interleukin-1beta increases expression and activity of matrix metalloproteinase-2 in cardiac microvascular endothelial cells: role of PKCalpha/beta1 and MAPKs. American Journal of Physiology. Cell Physiology. 2007; 292: C867–C875.

[20] Moriyama M, Ohno-Matsui K, Hayashi K, Shimada N, Yoshida T, Tokoro T, *et al*. Topographic analyses of shape of eyes with pathologic myopia by high-resolution three-dimensional magnetic resonance imaging. Ophthalmology. 2011; 118: 1626–1637.

[21] Ji F, Li Q, Zhu Y, Jiang L, Zhou X, Pan M, *et al*. Form deprivation myopia in C57BL/6 mice. [Zhonghua Yan Ke Za Zhi] Chinese Journal of Ophthalmology. 2009; 45: 1020–1026.

[22] Niu L, Zhang S, Wu J, Chen L, Wang Y. Upregulation of NLRP3 Inflammasome in the Tears and Ocular Surface of Dry Eye Patients. PloS one. 2015; 10: e0126277.

[23] Chen H, Zhang X, Liao N, Mi L, Peng Y, Liu B, *et al*. Enhanced Expression of NLRP3 Inflammasome-Related Inflammation in Diabetic Retinopathy. Investigative Ophthalmology & Visual Science. 2018; 59: 978–985.

[24] Tarallo V, Hirano Y, Gelfand BD, Dridi S, Kerur N, Kim Y, *et al*. DICER1 loss and Alu RNA induce age-related macular degeneration via the NLRP3 inflammasome and MyD88. Cell. 2012; 149: 847–859.

[25] Fledelius H, Zak M, Pedersen FK. Refraction in juvenile chronic arthritis: a long-term follow-up study, with emphasis on myopia. Acta Ophthalmologica Scandinavica. 2001; 79: 237–239.

[26] Herbort CP, Papadia M, Neri P. Myopia and inflammation. Journal of Ophthalmic & Vision Research. 2011; 6: 270–283.

[27] Fei J, Liang B, Jiang C, Ni H, Wang L. Luteolin inhibits IL-1β-induced inflammation in rat chondrocytes and attenuates osteoarthritis progression in a rat model. Biomedicine & Pharmacotherapy. 2019; 109: 1586–1592.

[28] Wang F, Guan M, Wei L, Yan H. IL 18 promotes the secretion of matrix metalloproteinases in human periodontal ligament fibroblasts by activating NF κB signaling. Molecular Medicine Reports. 2019; 19: 703–710.

[29] Zhao F, Wu H, Reinach PS, Wu Y, Zhai Y, Lei Y, *et al*. Up-Regulation of Matrix Metalloproteinase-2 by Scleral Monocyte-Derived Macrophages Contributes to Myopia Development. The American Journal of Pathology. 2020; 190: 1888–1908.

[30] Wu Y, He S, Bai B, Zhang L, Xue L, Lin Z, *et al*. Therapeutic effects of the artemisinin analog SM934 on lupus-prone MRL/lpr mice via inhibition of TLR-triggered B-cell activation and plasma cell formation. Cellular & Molecular Immunology. 2016; 13: 379–390.

[31] Wang L, Schmidt S, Larsen PP, Meyer JH, Roush WR, Latz E, *et al*. Efficacy of novel selective NLRP3 inhibitors in human and murine retinal pigment epithelial cells. Journal of Molecular Medicine (Berlin, Germany). 2019; 97: 523–532.

[32] Ahn H, Kang SG, Yoon S, Ko H, Kim P, Hong E, *et al*. Methylene blue inhibits NLRP3, NLRC4, AIM2, and non-canonical inflammasome activation. Scientific Reports. 2017; 7: 12409.