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Original Article

Increasing autophagy ameliorates all-*trans*-retinal-activated NLRP3 inflammasomes in human THP-1 macrophages

Qingqing Xia*, Jie Li*, Lingmin Zhang, Yingying Zhou, Zaixing Yang, Jing Xie

*Department of Laboratory Medicine, Huangyan Hospital of Wenzhou Medical University, Taizhou First People's Hospital, Taizhou, Zhejiang, China. *Equal contributors.*

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Abstract: Objective: Severe inflammation, mediated by innate immune sensors, can be observed in the retina and is considered to play an important role in the pathogenesis of retinal degeneration caused by all-*trans*-retinal (atRAL). However, the underlying mechanism thereof remains elusive. This study investigated the effects of atRAL on the macrophage cell line THP-1 and determined the underlying signaling pathway through pharmacological and genetical manipulation. Methods: The cytotoxicity of atRAL in THP-1 macrophage cells was assessed using the cell counting kit-8 (CCK-8) assay, and mature IL-1β was detected by enzyme-linked immunosorbent assay (ELISA). We measured levels of NLRP3 and cleaved caspase-1 by western blotting to evaluate the activation of NLRP3 inflammasomes. Oxidative stress was validated by measuring mitochondria-associated reactive oxygen species (ROS) with MitoSOX™ Red staining. Autophagy was assessed with the LC3BII turnover assay and tandem mCherry-eGFP-LC3B fluorescence microscopy. Results: The maturation and release of IL-1β were regulated by the activation of the NLRP3 inflammasome. Mitochondria-associated ROS were involved in the regulation of NLRP3 inflammasome activation and caspase-1 cleavage. In addition, atRAL functionally activated autophagy in THP-1 cells, and atRAL-induced NLRP3 inflammasome activation was suppressed by autophagy. Conclusions: atRAL activates both the NLRP3 inflammasome and autophagy in THP-1 cells, and the increasing level of autophagy leads to the inhibition of excessive NLRP3 inflammasome activation. These findings shed new light on the pathogenesis of age-related retinal degeneration.

Keywords: All-*trans*-retinal, NLRP3 inflammasome, autophagy, age-related macular degeneration, reactive oxygen species

Introduction

The eye has previously been considered as an immune privileged organ, but recent findings have suggested that inflammation that is mediated by innate immune sensors occurs in the retina [1]. Inflammasomes are critical innate immune components, and increasing evidence has suggested that the activation of the NLRP3 inflammasomes is involved in the development and progression of age-related macular degeneration (AMD) and Stargardt's disease [2-5]. The NLRP3 inflammasome is a large intracellular multimeric protein complex consisting of the NLRP3 receptor, the adaptor molecule apoptosis-associated speck-like protein containing a Caspase Activation and Recruitment Domain (CARD), and caspase-1 [6]. The NLRP3 inflammasome formation activates caspase-1, which proteolytically cleaves pro-interleukin (IL)-1β and pro IL-18 [6, 7]. Although research has demonstrated that the activation of NLRP3 inflammasomes was protective in neovascular AMD, its role in promoting pathogenesis of geographic atrophy has been documented [1, 2]. A previous clinical report indicated that up to 90% of advanced AMD was atrophic, suggesting that NLRP3 inflammasome activation could contribute to the pathogenesis of AMD [7].

All-*trans*-retinal (atRAL) (**Figure 1A**) is an important intermediate in the visual cycle, a complex enzymatic pathway for retinoid metabolism and regeneration within the retina. Allostatic over-

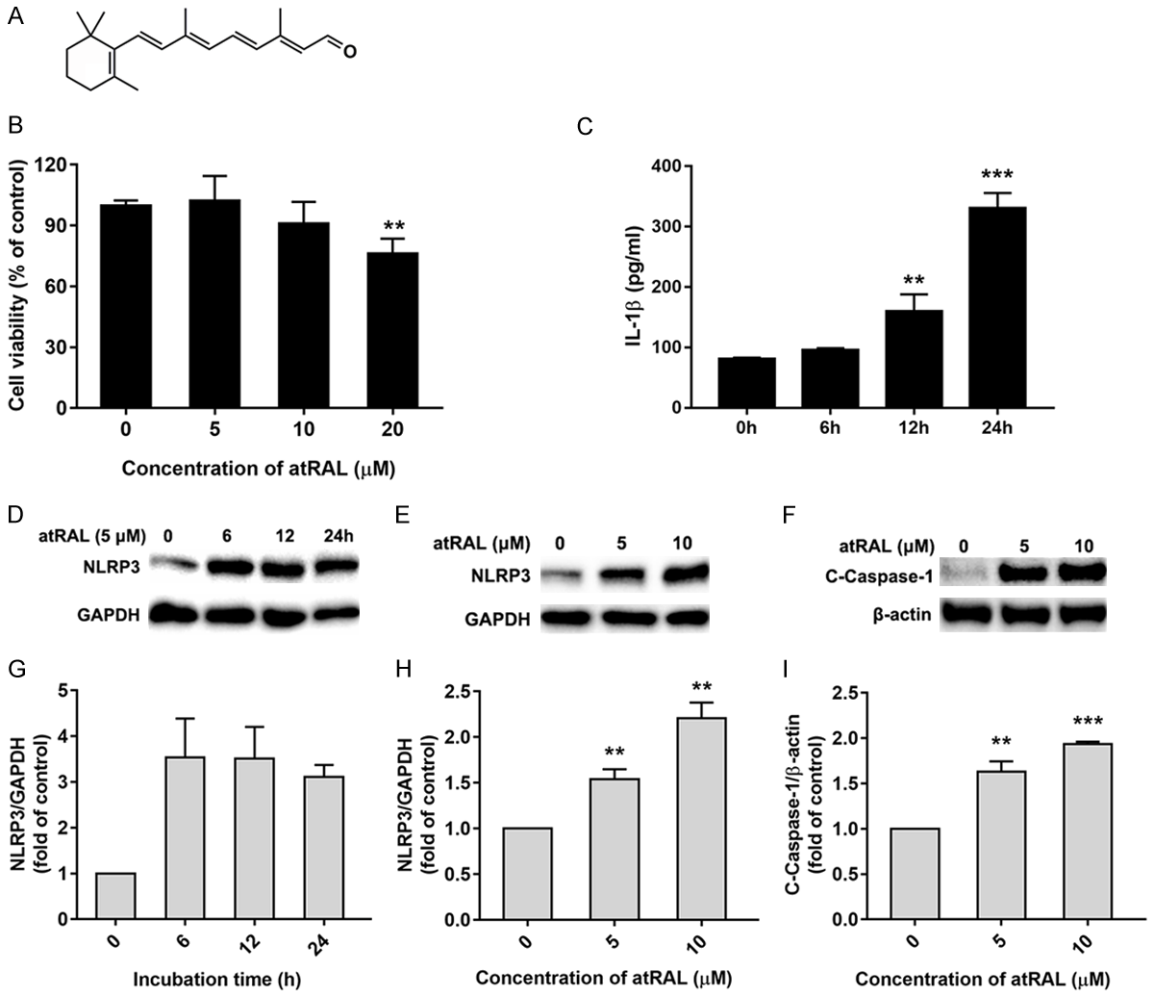

Figure 1. atRAL induces the release of IL-1β in THP-1 cells. (A) Structure of all-*trans*-retinal (atRAL). (B) Viability of THP-1 cells was measured using CCK8 after incubated with atRAL (0, 5, 10, 20 μM) for 24 h. n=6, **P<0.01. (C) Concentration of IL-1β in the cell culture medium was assayed by ELISA after the cells were exposed to atRAL for 0, 6, 12, or 24 h. n=3; **P<0.01; ***P<0.001. (D and G) After cells were treated with atRAL (5 μM) for 0, 6, 12, or 24 h, NLRP3 was determined by western blotting. Densitometric quantification of NLRP3 was analyzed with Image Lab™ software (n=3). (E and H) NLRP3 and (F and I) cleaved-caspase 1 (C-caspase 1) were detected by western blotting after THP-1 cells were incubated with atRAL (0-10 μM) for 24 h. Densitometric quantification of NLRP3 and C-caspase 1 was analyzed with Image Lab™ software (n=3). The expression of these proteins was compared relative to GAPDH or β-actin. **P<0.01 and ***P<0.001.

load, which can be caused by aging, genetic predisposition, or environmental factors, disrupts the visual cycle, leading to the progression of AMD [5]. Thus, atRAL clearance failure is associated with retinal degeneration [8-11].

Previous studies have demonstrated that delayed atRAL clearance after light exposure can cause the sub-retinal infiltration of microglia/macrophages [8], indicating that the resident or infiltrating immune cells may be involved in atRAL-induced inflammation of the retina. It has also been found that atRAL-induced photoreceptor apoptosis can result in double-stranded RNA or protein release, which in turn activates toll-like receptor (TLR)3 and TLR4 [8, 10], respectively. These findings suggest that immunoregulatory mechanisms are implicated in the pathogenesis of atRAL-induced retinal degeneration. The NLRP3 inflammasome has been foundto be involved in sensing endogenous danger signals, including uric acid crystals, extracellular ATP, and fatty acids [12-14]. In the present study, we hypothesized that atRAL could activate NLRP3 inflammasomes in the macrophage cell line THP-1. We found that

the maturation and release of IL-1β was regulated by the caspase-1 pathway, and the activation of the NLRP3 inflammasome was essential for the release of IL-1β. Further, mitochondria-associated reactive oxygen species (ROS) were involved in the regulation of the NLRP3 inflammasome activation and caspase-1 cleavage. In addition, atRAL functionally activated autophagy in the THP-1 cells, and the NLRP3 inflammasome activation was inhibited by enhancing autophagy. Collectively, these results demonstrated the role of atRAL in the NLRP3 inflammasome activation and elucidated its underlying mechanism.

Materials and methods

Reagents and cell line

atRAL and trehalose were purchased from Sigma-Aldrich (St. Louis, MO, USA), and atRAL was dissolved in dimethyl sulfoxide as a stock solution (20 mM) and kept in the dark. The caspase-1 inhibitor Z-YVAD-FMK was obtained from Abcam (UK), and 3-methyladenine (3-MA) was obtained from Selleck Chemicals (Shanghai, China). Chloroquine (CQ) was obtained from Solarbio (Beijing, China), and the antioxidant N-Acetyl-L-cysteine (NAC) was purchased from Beyotime Biotechnology (Shanghai, China). The primary antibodies, rabbit anti-β-actin and mouse anti-GAPDH, were obtained from Proteintech Company (USA); rabbit anti-LC3B, rabbit anti-NLRP3, and rabbit anti-cleaved caspase-1 were provided by Cell Signaling Technology (USA).

The human acute monocytic leukemia cell line THP1 was provided by the cell bank of the Chinese Academy of Sciences (Shanghai, China). The THP1 cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin in a humidified incubator at 37°C and 5% CO_2. The THP1 cells were seeded in 6- or 96-well cell culture plates (Corning, Shanghai) and stimulated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) for 48 h prior to subsequent experiments.

Analysis of cell viability

Cells were treated with atRAL (0-20 μM) for 24 h, and then the cell viability was examined using the cell counting kit-8 (CCK8) (Dojindo, Japan) assay. The culture supernatant in each well was aspirated, then 10 μL of CCK8 and 90 μL of medium was added to the cells and incubated for 1 h at 37°C. The absorbance was measured at 450 nm in each well with a microplate spectrophotometer (Multiskan FC, Thermo Scientific, USA). Cell viability was presented as a proportion of the control optical density.

Enzyme-linked immunosorbent assay (ELISA)

The concentration of IL-1β in the cell culture supernatant was evaluated using an IL-1β ELISA Kit (Invitrogen, USA) according to the manufacturer's instructions.

Measurement of mitochondria-associated ROS in THP1 macrophages

Mitochondrial superoxide, which is generated as a by-product of oxidative phosphorylation, is the predominant ROS in mitochondria [15]. In the present study, mitochondrial superoxide within the THP1 macrophages was measured using MitoSOX™ red (Invitrogen Molecular Probes, USA) staining. Briefly, the THP1 cells were challenged with 5 μM atRAL for 24 h, and then incubated with 2.5 μM MitoSOX™ Red solution in Dulbecco's Modified Eagle Medium at 37°C for 10 mins. The cells were rinsed three times with PBS, and were subsequently observed and photographed using an inverted fluorescence microscope (Olympus, Japan).

siRNA transfection

NLRP3 siRNA (5'-GUAAGCCUAGGUGUUGUCATT-3') and a negative control (NC) siRNA (5'-GCGACGAUCUGCCUAAGAUdTdT-3') were purchased from GenePharma Company (Shanghai, China). The *NLRP3* siRNA or NC siRNA (100 nM) transfection solutions were prepared with Lipofectamine 3000 (Invitrogen) and Opti-MEM™ (Gibco) according to the manufacturer's instructions. The THP-1 cells were incubated with the siRNAs for 6 h and cultured in complete medium for another 24 h.

Autophagic flux monitoring

Autophagic flux in the THP-1 macrophage cells was monitored by tandem mCherry-eGFP fluorescence microscopy. Briefly, THP1 cells were infected with 1 μL of the recombinant adenovirus (3.2×10^{10} pfu/mL) carrying a tandem

monomeric mCherry-eGFP-tagged LC3B (Vigene Biosciences, Shandong, China) for 24 h. The cells were exposed to atRAL for a further 24 h. After the culture supernatant was replaced with fresh medium, and the cells were observed and analyzed using fluorescence microscopy (Olympus).

Western blot analysis

Cell lysates were prepared with a RIPA lysis buffer (Beyotime) containing 1% protease inhibitor cocktail (Hangzhou Fude Biological Technology, China). Cell protein concentration was determined using an enhanced BCA protein assay kit (Beyotime). Subsequently, each sample was combined with an appropriate volume of loading buffer and boiled for 5 min with an intelligent constant temperature metal bath. The proteins were subjected to gel electrophoresis with SDS-PAGE denaturing gels and transferred to a PVDF membrane by electroblotting (Millipore, USA). Membranes were blocked with 5% skimmed milk at room temperature for 1 h. Each membrane was incubated with primary antibodies at 4°C overnight. Membranes were then rinsed three times with the wash buffer, Tris-buffered saline with Tween-20 (TBST) and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Beyotime) at room temperature for 1 h. The protein bands were visualized using the Clarity™ Western ECL Substrate (Bio-Rad, USA). The ChemiDox™ XRS + system (Bio-rad) and Image Lab™ software (Bio-Rad) were used for the determination and analysis of all western blot results.

Statistical analysis

All data in the present study were expressed as the mean ± standard error of the mean. For statistical analysis, one-way analysis of variance followed by Tukey's test for multiple comparisons was used for data containing more than two groups. All data were analyzed using GraphPad Prism software (version 7.0; San Diego, CA, USA), and a $P<0.05$ was considered significant.

Results

atRAL induces the release of IL-1β in THP-1 cells

We first examined atRAL-induced cytotoxicity in the THP-1 cells. As depicted in **Figure 1B**, the test concentrations of 5 or 10 μM atRAL appeared to produce no cytotoxicity in the THP1 cells, whereas at the concentration of 20 μM, the cell viability was significantly reduced after treatment for 24 h. To avoid the cytotoxic effects induced by high doses, we used 5 μM of atRAL in the subsequently experiments. We observed that the expression of NLRP3 (**Figure 1D** and **1G**) and resulting release of IL-1β (**Figure 1C**) were significantly increased by 5 μM atRAL treatment within 24 h. Also, THP-1 cells exposed to 5 or 10 μM of atRAL demonstrated a dose-dependent increase in the expression of NLRP3 (**Figure 1E** and **1H**) and activation of caspase-1 (**Figure 1F** and **1I**). These findings suggest that atRAL can cause an increase in NLRP3 and activation of caspase-1, and consequently the production of mature IL-1β.

NLRP3 inflammasome activation is implicated in atRAL-induced IL-1β maturation

Activated caspase-1 is the key regulatory component of the inflammasome multiprotein complex responsible for the processing of pro-IL-1β into the mature IL-1β [6]. We therefore evaluated the role of caspase-1 in the atRAL-induced maturation of IL-1β using the caspase-1 inhibitor Z-YVAD-FMK. It was found that incubation with Z-YVAD-FMK (1, 5, and 10 μM) significantly decreased the expression of cleaved caspase-1 (**Figure 2A** and **2F**) and attenuated the maturation of IL-1β (**Figure 2B**). This suggests that atRAL-induced IL-1β production is mediated by caspase-1 regulation. We further genetically knocked down NLRP3 using a specific siRNA and found that the NLRP3-targeted siRNA dramatically diminished the NLRP3 protein levels (**Figure 2C** and **2G**) and abolished atRAL-induced cleaved caspase-1 expression (**Figure 2D** and **2H**). Moreover, we found that silencing *NLRP3* suppressed the maturation and release of IL-1β induced by atRAL in THP-1 cells (**Figure 2E**). Taken together, these data indicate that atRAL exposure may induce NLRP3 inflammasome activation in THP-1 cells.

atRAL-induced ROS generation is involved in NLRP3 inflammasome activation

Mitochondrial ROS production was found to be important in the NLRP3 inflammasome activation, so we determined whether the mitochondrial ROS was generated in response to atRAL treatment in the THP-1 cells. As shown in **Figure**

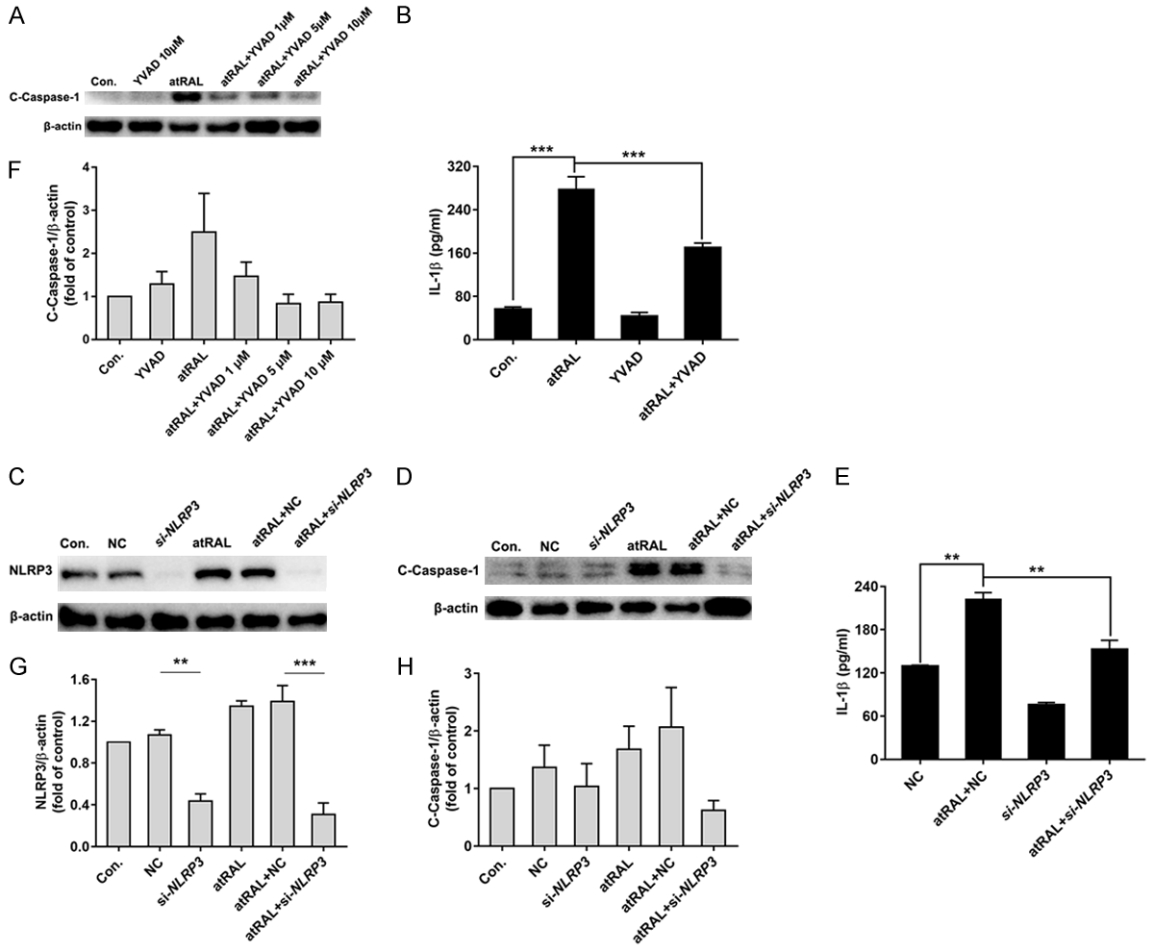

Figure 2. NLRP3 inflammasome activation is implicated in atRAL-induced IL-1β maturation. A and F. C-caspase 1 was detected by western blotting after the cells were incubated with or without atRAL (5 μM) in the presence or absence of 1, 5, or 10 μM YVAD for 24 h. Densitometric quantification of C-caspase 1 was analyzed with Image Lab™ software (n=3). B. The effect of NLPR3 inhibitor YVAD on the production of IL-1β in the cell culture medium. n=3; ***P<0.001. C and G. The knockdown efficiency of si-*NLRP3* on the expression of NLRP3 in the presence or absence of atRAL. Densitometric quantification of NLRP3 was analyzed with Image Lab™ software (n=3). **P<0.01 and ***P<0.001. D and H. The effects of suppressing NLRP3 by siRNA on the activation of C-caspase 1 (p20) in the presence or absence of atRAL. Densitometric quantification of C-caspase 1 was analyzed with Image Lab™ software. Expression of these proteins was compared relative to β-actin. n=3; **P<0.01 and ***P<0.001. E. The effect of NLPR3 interference by siRNA on the production of IL-1β in the cell culture medium. n=3; **P<0.01.

3A and **3D**, exposure of the THP-1 macrophages to atRAL significantly increased the generation of mitochondria-associated ROS. Treatment with NAC, a frequently used ROS scavenger, significantly inhibited the accumulation of NLRP3 in the THP-1 cells after atRAL exposure (**Figure 3B** and **3E**). In addition, we found that NAC could diminish the expression of cleaved caspase-1 (**Figure 3C** and **3F**). These data suggest that the activation of the NLRP3 inflammasomes requires atRAL-induced mitochondrial ROS overproduction by THP-1 cells.

atRAL-induced NLRP3 inflammasome activation is suppressed by autophagy

LC3BII is frequently used as a marker for autophagosomes and is converted from LC3BI during autophagy. We therefore utilized it to determine whether autophagy plays a role in the activation of NLPR3 inflammasomes induced by atRAL. We found that incubation with atRAL (5 μM) significantly increased the expression of LC3BII after 6 h, with the expression level remaining stable from 12 to 24 h

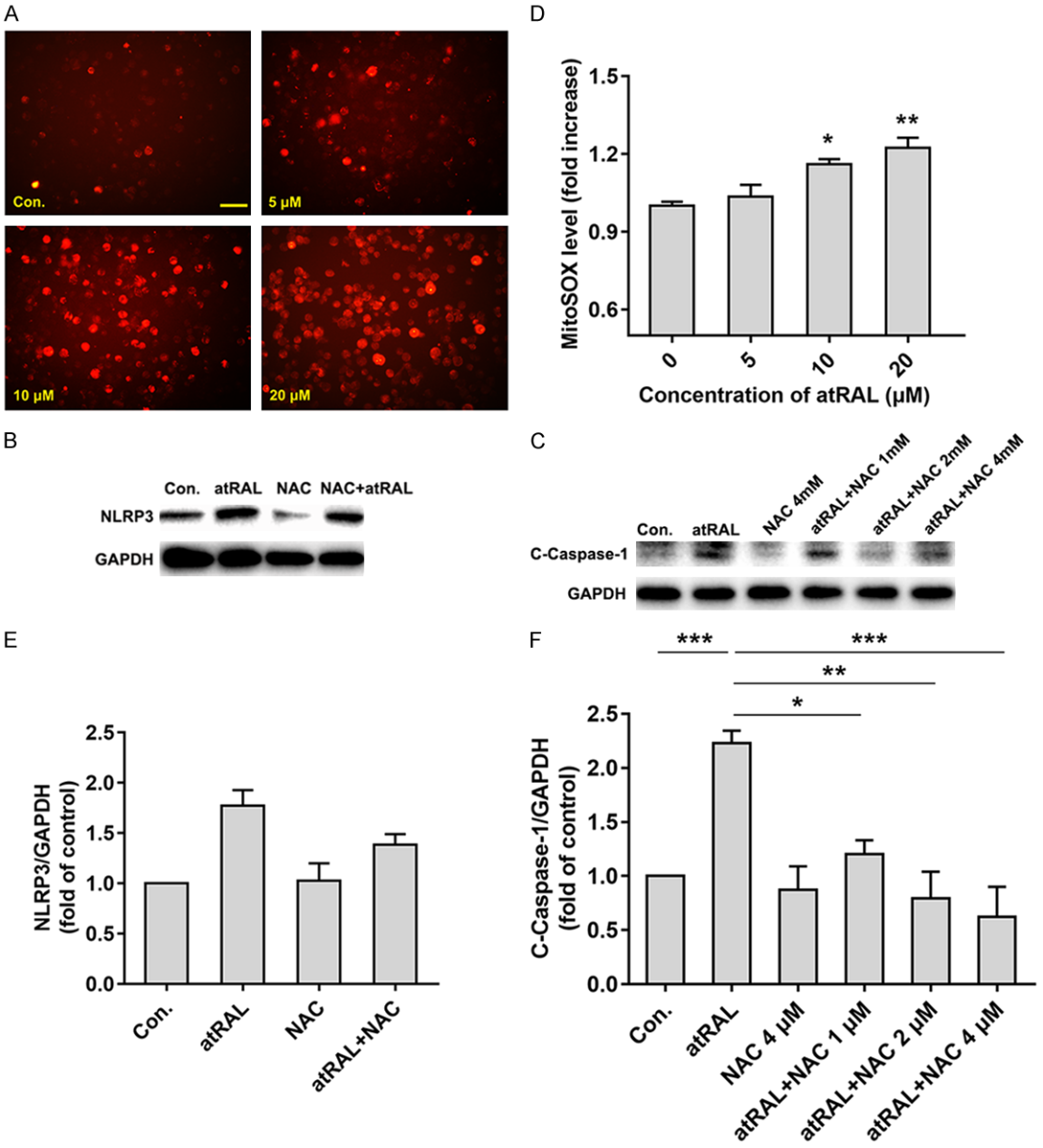

Figure 3. atRAL-initiated ROS generation is involved in NLRP3 inflammasome activation. A and D. After THP-1 cells were incubated with atRAL (0, 5, 10, 20 μM) for 24 h, the mitochondrial ROS were measured with MitoSOX™ red staining and visualized by fluorescence microscopy. Con., Control, Scale bar, 50 μm. Densitometric quantifications of red signal was achieved with ImageJ (n=3). **P<0.01. B and E. NLRP3 was detected after treatment with atRAL (5 μM) in the presence or absence of NAC (2 mM). Densitometric quantification of NLRP3 was analyzed with Image Lab™ software (n=3). C and F. C-caspase 1 was evaluated after treatment with atRAL (5 μM) in the presence or absence of NAC (1, 2, and 4 mM). Densitometric quantification of C-caspase 1 was analyzed with Image Lab™ software. Expression of these proteins was compared relative to β-actin. n=3; *P<0.05, **P<0.01 and ***P<0.001.

(**Figure 4A** and **4C**). The LC3B turnover assay was utilized to further examine atRAL-induced autophagy in THP-1 cells. Our data showed that atRAL-induced LC3BII accumulation could be further enhanced with CQ treatment (**Figure 4B** and **4D**). Furthermore, tandem mCherry-eGFP fluorescence microscopy was used to monitor the autophagic flux [16]. The THP-1 cells were infected with the recombinant adeno-associated virus vectors carrying tandem sensor

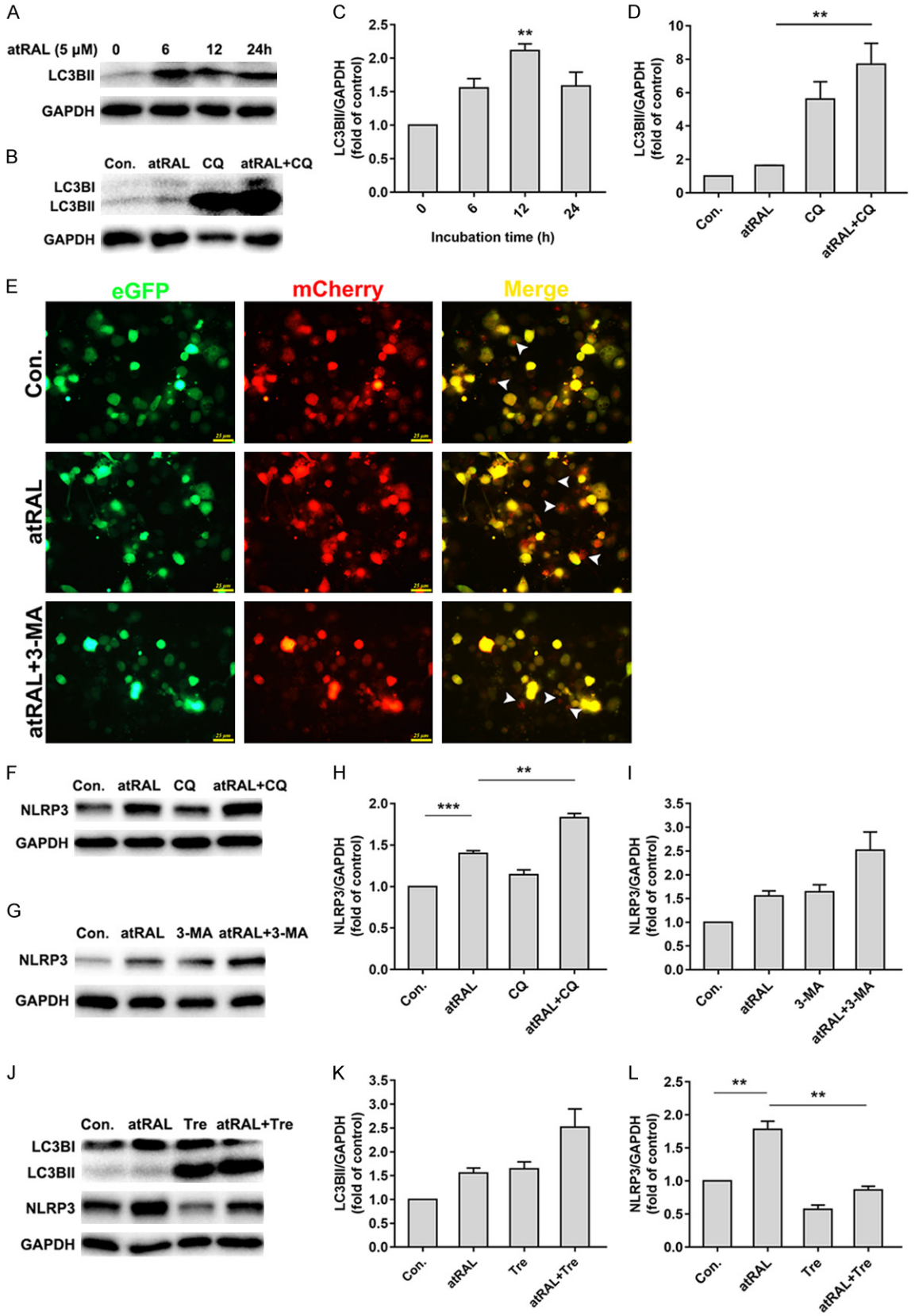

Figure 4. atRAL-caused NLRP3 inflammasome activation is suppressed by autophagy. A and C. LC3BII was determined by western blotting after THP-1 cells were treated with atRAL (5 μM) for 0, 6, 12 h. Densitometric quantifica-

tions of LC3BII was analyzed with Image Lab™ software (n=3). **P<0.01. B and D. After THP-1 cells were incubated with atRAL (5 μM) for 24 h in the presence or absence of 20 μM CQ, LC3BII was measured for autophagy monitoring. Densitometric quantifications of LC3BII was analyzed with Image Lab™ software (n=3). **P<0.01. E. Recombinant adeno-associated virus vectors carrying mCherry-EGFP-LC3B were incubated with THP-1 cells for 24 h. Cells were then exposed to 5 μM atRAL for 24 h and analyzed by fluorescence microscopy. Con., Control, Scale bar, 25 μm. F-I. Cells were incubated with 5 μM atRAL for 24 h in the absence or presence of 20 μM CQ or 5 mM 3-MA, and immunoblotted for NLRP3. Densitometric quantifications of NLRP3 was analyzed with Image Lab™ software (n=3). **P<0.01; ***P<0.001. J-L. After cells were incubated with 5 μM atRAL for 24 h in the absence or presence of 100 μM Tre, LC3B, and NLRP3 were evaluated with immunoblotting. Densitometric quantifications of LC3BII and NLRP3 were analyzed with Image Lab™ software (n=3). **P<0.01.

mCherry-eGFP-LC3B constructs. The eGFP is easily degraded in the lysosome, but the mCherry is more stable under low pH and/or proteolytic conditions. Thus, autolysosomes exhibit only a red signal under fluorescence microscope, whereas yellow fluorescence indicates a phagophore or autophagosome that has failed to fuse with the lysosome. We found that atRAL exposure elevated the number of cells with more puncta in red (**Figure 4E**, white arrow heads). However, 3-MA, a classic autophagy inhibitor, significantly suppressed the increase in red fluorescence in the THP-1 cells (**Figure 4E**, white arrow heads). We also observed that the autophagy inhibitors CQ or 3-MA augmented atRAL-induced NLRP3 expression (**Figure 4F-I**). Moreover, the novel autophagy activator, trehalose (Tre), upregulated the expression of atRAL-induced LC3BII and suppressed the NLRP3 accumulation (**Figure 4J-L**). These data indicate that atRAL activates autophagy, which may inhibit NLRP3 inflammasome activation in THP-1 cells.

Discussion

Aging, gene defects, or the accumulation of certain environmental factors may contribute to the failure of atRAL clearance, resulting in atRAL accumulation and causing cytotoxicity in the retina [17, 18]. Under these circumstances, retinal pigment epithelium (RPE), photoreceptor cells, microglia, or macrophages may be exposed to free atRAL. Accumulating evidence demonstrates that danger signals such as *Alu* RNA, A2E, and atRAL, can activate the NLRP3 inflammasomes in RPE [1, 3, 19]. However, a recently reported study argued that there may be negligible expression of NLRP3 in RPE cells, suggesting minimal contributions for the activation of the NLRP3 inflammasomes [20]. These results indicate that the intra-retinal immune cells, such as macrophages, either resident or infiltrating, rather than the RPE cells, are implicated in the detrimental effect of atRAL in retinal degeneration. Therefore, the present study used PMA-differentiated THP-1 macrophage cells, which are widely used in NLRP3 inflammasome research [21], to verify whether atRAL exposure activates the NLRP3 inflammasomes.

Excessive concentrations of free atRAL in the retina can lead to severe cytotoxicity due to its highly reactive aldehyde, which may directly react with biological macromolecules [9]. Our results demonstrated this when an atRAL dose of 20 μM not only led to the pathogenesis effect of atRAL on AMD (low-dose effect), but also caused immunotoxicity in the retina (high-dose effect). We found that atRAL treatments increased the maturation and release of IL-1β and upregulated the expression of NLRP3 and cleaved caspase-1 in the macrophage THP-1 cell line in a time-dependent manner. These findings corroborate previously reported data that free atRAL may create a proinflammatory microenvironment in the retina and contribute to the pathogenesis of AMD [3, 19]. By using the caspase-1 inhibitor, Z-YVAD-FMK, and knockdown of NLRP3, we clarified the underlying mechanism, i.e., NLRP3 recruits ASC and procaspase-1 in response to cellular stress to form the NLRP3 inflammasome, resulting in caspase-1 activation, and IL-1β maturation [22]. The present study confirmed that the accumulation of atRAL may activate NLRP3 inflammasomes in macrophage cells and contribute to the para-inflammation or chronic inflammation in the retina [23].

Accumulating evidence has suggested that ROS play a positive role in NLRP3 inflammasome activation [12, 21, 22], which is consistent with the present study, as our data demonstrated that the ROS quencher NAC suppressed NLRP3 expression and caspase-1 cleavage. ROS are verified to be early inducers of autoph-

agy [11]. This study found that the autophagic flux was elevated following atRAL-incubation. It is well recognized that autophagy activation contributes to NLRP3 degradation and inhibits IL-1β expression [24]. Consistently, we found that atRAL-induced autophagy suppressed NLRP3 expression levels, suggesting that atRAL simultaneously activates NLRP3 inflammasomes and autophagy in THP-1 cells, and the latter inhibits the excessive activation of NLRP3 inflammasomes.

A previous study demonstrated that atRAL could activate caspase-3/gasdermin E (GSDME)-mediated pyroptosis, which was accompanied by the activation of the NLRP3 inflammasome and elevation of IL-1β secretion in RPE cells [3]. Another recent work emphasized that GSDME-induced cell death played a role in atRAL-mediated retina damage [25], which strengthens the idea that repressing pyroptosis may be a treatment for photoreceptor atrophy in dry AMD and Stargardt's disease. Our results are consistent with these reported data.

However, there are several limitations in this research. First, though THP1 cells are widely used macrophage model, they are a kind of leukemia cells. Our results need to be further verified using other macrophages such as primary murine bone marrow-derived macrophages or peritoneal macrophages in the future. Second, the role of NLRP3 inflammasome activation in the pathogenesis of dry AMD is still unclear, thus further animal experiments with *Abca4*$^{-/-}$*Rdh8*$^{-/-}$ mice are needed to verify these findings.

Conclusions

atRAL may activate the NLRP3 inflammasome and accelerate the secretion of IL-1β in intraretinal immune cells. Mitochondrial ROS generation plays an important role in the atRAL-induced NLRP3 inflammasome activation. Moreover, atRAL-related autophagy suppresses NLRP3 inflammasome activation in the retina. This work provides further insight into atRAL-associated retinopathy.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Jing Xie and Zaixing Yang, Department of Laboratory Medicine, Huangyan Hospital of Wenzhou Medical University, Taizhou First People's Hospital, Taizhou, Zhejiang, China. Tel: +86-0576-84016508; E-mail: jingxietzsy@163.com (JX); yangzaixingdiyi@163.com (ZXY)

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