NF-κB/ABCA1 pathway aggravates ox-LDL-induced cell pyroptosis by activation of NLRP3 inflammasomes in THP-1-derived macrophages

Jiashan Li1,3 · Jiaru Liu1,3 · Ying Yu1,3 · Yuee Liu1,3 · Xiuru Guan2,3

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Abstract
Objective NF-κB (nuclear transcription factor-kappa B) plays a well-known function in the regulation of immune responses and inflammation, but growing evidences support a major role of it in atherosclerosis. Currently, the regulatory mechanism of NF-κB pathway involved in atherosclerosis remains unclear.

Methods To investigate the role of ox-LDL (oxidized low-density lipoprotein) in NF-κB regulation, the protein expression of phosphorylated NF-κB, a marker of NF-κB pathway activation was measured. The pyroptosis of macrophage was evaluated by western blot and fluorescence microscope. Cholesterol efflux capacity was determined by fluorescence assay and oil red O staining. The inhibitor of activation of NF-κB signal was used to assess the effect of NF-κB signal on macrophage pyroptosis and cholesterol efflux in macrophage. Small interfering RNA of ABCA1 (cholesterol transporters ATP binding boxes A1) was used to assess the effect of ABCA1 on macrophage pyroptosis.

Results In this study, we reported THP-1 derived macrophage can be stimulated to increase pyroptosis by ox-LDL in a concentration-dependent manner. Macrophage pyroptosis was correlated with enhanced activation of NF-κB signal. After using inhibitor of NF-κB phosphorylation to attenuate activation of NF-κB signal, we identified and confirmed the decrease of macrophage pyroptosis and the occurrence of ox-LDL-induced cholesterol efflux disorder. Furthermore, we found that the downregulation of ABCA1 led to increased cell inflammation death. But pyroptosis was blocked, may led to cholesterol efflux dysfunction.

Conclusion Taken together, the present results indicate that the mechanism of NF-κB involved in the development of atherosclerosis depends on mediating cell pyroptosis and cholesterol efflux and provide significant light on macrophage NF-κB signal in atherosclerosis.

Keywords NF-κB signal · ABCA1 · Atherosclerosis · Cell pyroptosis

Introduction
Atherosclerosis (AS), a chronic inflammatory disease of artery blood vessels, represents the primary cause of morbidity and premature death worldwide. Its pathogenesis, however, is still unclear now. Recent studies have focused on pyroptosis, a unique form and mechanism of programmed cell death compared with apoptosis and necrosis, and characterized by the release of large amounts of inflammatory mediators, which has been shown to be associated with atherosclerotic diseases [1, 2]. Mechanistically, the process of pyroptosis is well preserved across cell types, and characterized by activation of NLRP3 (nucleotide-binding domain leucine-rich repeat (NLR) and pyrin domain containing receptor 3) inflammasomes. Activated NLRP3 transduces the recognition signal...
Materials and methods

Materials

THP-1 mononuclear macrophage cell lines were selected from Beijing Beina Institute of Biotechnology (BNCC). GSDMD, P-IkB, P-NFκB, β-actin primary antibody, cholesterol efflux fluorescence detection kit and sheep anti-mouse IgG secondary antibody were all from Abcam. Skim milk powder from BD company; NLRP3 primary antibody from Cell Signaling Technology Inc.; Human IL-1β enzyme-linked immunosorbent assay kit from Elabscience; RPMI 1640 and Fetal Bovine Serum from Hyclone; PMA comes from Sigma; ECL luminescent liquid from Beijing Noblad Technology Co., Ltd. Oil red O dye from Beijing Solaibao Biological Co., Ltd. The sheep anti-rabbit IgG secondary antibody was obtained from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. OX-LDL from Guangzhou Yiyuan Biotechnology Co., Ltd. Color predyeing protein marker from the United States Thermo Company; HoechST33342/PI double dyeing kit and lactate dehydrogenase (LDH) assay kit were from Nanjing Jiancheng Institute of Biological Engineering; Cell Counting Kit-8 (CCK-8) was obtained from Tongren Chemical Research Institute, Japan; Bay11-7082 and VX-765 from Shanghai Taosu Biochemical Technology Co., Ltd. SIABCA1 transfection reagent package was obtained from Suzhou Genepharma gene. BCA protein quantitative kit is from China Biyuntian Biotechnology Co., Ltd.

Method

Cell treatment

THP-1 monocytes were purchased from the BeNa Culture Collection and cultured in RPMI1640 medium containing 10% fetal bovine serum (FBS) (both from Hyclone USA) at 37 °C and 5% CO₂. The cells were transferred to 6-well at a cell density of about 5 × 10⁵. Each well was incubated with PMA (Sigma, USA) at a final concentration of 100 ng/mL for 48 h to induce the differentiation of THP-1 cells into macrophages before being treated with the following: ox-LDL (0, 25, 50, 75, or 100 mg/mL) for 24 h; or BAY11-7082 (10 μM), VX-765 (50 μM) for 2 h.
Western blot analysis

The total protein was extracted using lysis buffer containing the protease inhibitor PMSF (Beyotime, China), and the protein concentration was measured using the BCA kit (Solarbio, China). The denatured proteins were separated using SDS–polyacrylamide gel electrophoresis, transferred to PVDF membranes, and then blocked with 5% bovine serum albumin (BSA) for 1 h. Target bands were incubated with the primary antibody overnight at 4 °C and then incubated with AP or HRP-conjugated secondary antibody for 1 h. The immune complexes were detected in an exposure apparatus using an enhanced chemiluminescence agent, and the target strip protein content was quantified using Tanon Gis software. The dilution ratios for the various antibodies were as follows: ABCA1 (1:500), NLRP3 (1:500), GSDMD (1:500), p-NFκB (1:1000), p-IκB (1:1000), b-actin (1:6000), goat anti-mouse secondary antibody (1:10,000), and goat anti-rabbit secondary antibody (1:50,000).

Cholesterol efflux detection

Macrophages were treated with the fluorescence labeling reagent according to the manufacturer’s kit instructions in 96-well plates for 16 h. After discarding labeling reagent, the cells were gently washed with serum-free RPMI medium and then treated with cholesterol receptor for 4–6 h. Then the supernatant was collected, 100 µl cell lysis buffer was added to each well and then incubated on a shaker for 30 min to dissolve the cells. The fluorescence intensity of the supernatant and lysate were measured at an Ex/Em of 482/515 nm. The fluorescence intensity of the supernatant was divided by the total fluorescence intensity of the supernatant and lysate, and the value was multiplied by 100 to obtain a % cholesterol efflux rate.

Oil red O staining

The supernatant in the six-well plate was aspirated, and then the cells were washed three times with PBS and treated with 4% paraformaldehyde for 6 min. Then remove the paraformaldehyde solution and wash with PBS for three times. Add the prepared dilute solution of oil red O to the cells at 37 °C for 15 min. Then remove oil red O and wash cells with PBS for three times. And then you have 60% isopropanol for 30 s, and washed another three times with PBS for microscopic observation and photography. The PBS in the plate was discarded and the dye was extracted with 100% isopropyl alcohol for 10 min. The OD value of the mixture was measured at 520 nm for quantitative analysis.

Enzyme linked immunosorbent assay

An appropriate amount of cell culture medium was taken, centrifuged at 1000 g for 20 min, and the supernatant was reserved at −20 °C for later use. First set standard hole and blank hole to draw standard curve. Incubate the remaining Wells with 100 µl of sample at 37 °C for 1 h, then remove the sample. Each well was quickly added with working solution A and incubated at 37 °C for 1 h. Repeat washing the board three times and removing. Then, each well was quickly added with working solution B and incubated at 37 °C for 30 min. Again wash the plate 5 times and try to drain liquid in the hole. TMB 90 µl was added into the corresponding well and incubated at 37 °C for 15 min. Add 50 µl termination solution to the corresponding hole, and record the O.D of each hole at 450 nm wavelength value.

Determination of LDH (lactate dehydrogenase) content

Three groups of duplicate holes were set up in the 96-well plate, respectively, for the control hole and the sample hole. 5 µl of double distilled water was added to each control well, and 20 µl of sample and 25 µl of matrix buffer were added to each control well and test well. Finally, 5 µl coenzyme I was added to the experimental well, and the plate was mixed and incubated at 37 °C for 15 min. After that, 25 µl of 4-dinitrophenylhydrazine were added to each well at 37 °C for 15 min. After discarding liquid, 250 µl of 0.4 mol/L NaOH solution was added to each well at room temperature for 5 min. At the wavelength of 450 nm, the absorbance was measured with a microplate analyzer.

Hoechst33342/PI double staining test

The adherent cultured cells were digested and collected by trypsin, then the trypsin was removed by centrifugation, and 1 mL serum-free medium and 10 µl HoechST33342 dye solution were added to each well, mixed, and incubated at 37 °C for 5–15 min. Then centrifuge at 4 °C at 800 RPM for 5 min to discard the waste liquid; Add 1 mL Buffer A working solution, suspend cells and add 5 µl PI dye and avoid light at room temperature for 5–15 min. Finally, fluorescence microscope and flow cytometry analysis: PI produced red fluorescence.

CCK8 cell activity assay

After cells were treated, the original medium was discarded, 100 µl Cell Counting Kit-8 reagent of 10 mg/mL was added to each well, and the cells were incubated at
With the increase of ox-LDL concentrations, macrophage-derived foam cells pyroptosis and NF-κB signal activation both increased a CCK8 assay for cell viability; b the expression of NLRP3, GSDMD and p-NFκB and p-IκB protein were detected by Western Blot after treatment with ox-LDL concentration gradient (0, 25, 50, 75, 100 μg/mL), and β-actin was used as internal reference. c–f Statistical graph of relative gray values of protein bands; g ELISA was used to detect the content of IL-1β in the supernatant of culture medium; h The content of LDH in medium supernatant was detected and analyzed statistically; i fluorescence microscopy analysis after Hoechst33342/PI double staining; the scale is 50 μm. Among them, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, n = 3

37 °C for 4 h. Measure the optical density (OD) value at 450 nm. Each experiment was repeated three times.

Statistical analysis

Data were processed using GraphPad Prism 9 software and are expressed as mean ± standard deviation. The t-test was performed for two-group comparisons, and comparisons of three groups or more were analyzed by one-way analysis of variance. Differences were considered statistically significant at *p < 0.05.

Results

With the increase of ox-LDL concentrations, macrophage-derived foam cells pyroptosis and NF-κB signal activation both increased

To test the effect of ox-LDL on the survival rate of macrophages, THP-1-derived macrophages were treated with a range of concentrations of ox-LDL for 24 h. We found cell viability decreased with the increase of ox-LDL concentration (Fig. 1a). The pyroptosis of THP-1-derived cells was gradually induced by the increase of ox-LDL concentration as shown by the increased expression of pyroptosis marker protein NLRP3 inflammasome and GSDMD (Fig. 1b–d), the increased release of mature IL-1β and LDH in the culture supernatant (Fig. 1g, h), and the increase of the proportion of Hoechst33342/PI-stained double positive cells (Fig. 1i). Additionally, we found that the expressions of p-NF-κB and p-IκB proteins were increased, indicating the increased activation of NF-κB signal in a concentration-dependent manner of ox-LDL in macrophages (Fig. 1e, f).

NF-κB phosphorylation inhibitors blocked ox-LDL-induced cell pyroptosis and enhanced ABCA1 protein expression

BAY11-7082, a specific inhibitor of NF-κB phosphorylation, was cultured with THP-1-derived macrophages at 10 μM for 2 h, and then 50 μg/mL ox-LDL was added (according to Fig. 1), 50 μg/mL ox-LDL significantly induced pyroptosis compared with the untreated control group and decreased cell toxic death compared with 75/100 μg/mL ox-LDL, so in the following experiments, the macrophages were treated with 50 μg/mL ox-LDL for 24 h). We found that the inhibitor was effective as shown by the significantly decreased expression of p-NFκB in the experimental group (Fig. 2a, b). Meanwhile, the protein expression of NLRP3 and GSDMD as well as the IL-1β contents in the medium (Fig. 2e) were also significantly decreased (Fig. 2a, c, d). And the results of Hoechst33342/PI double staining and LDH release assay also showed that the formation of cell membrane pores and the release of cellular contents reduced (Fig. 2f, j). In summary, we found that inhibition of NF-kB signal activation could significantly block ox-LDL-induced cell pyroptosis.

Surprisingly, ox-LDL may inhibit ABCA1-regulated reverse cholesterol transport by activating NF-κB signal as shown by increased ABCA1 expression (Fig. 2g, h) and cholesterol efflux (Fig. 2i) under BAY11-7082 treatment, which significantly reversed the inhibition of ox-LDL treatment on the expression of ABCA1 proteins and ABCA1-regulated cholesterol efflux.

Dysfunction of ABCA1-regulated cholesterol efflux increases cell pyroptosis

First, the three kinds of SiABCA1 were mixed with transfection reagents for treatment of macrophages for 5 h, and siABCA1 (a) with the best inhibition effect was selected by Western Blot (Fig. 3a, b). Therefore, in the following experiment, si-ABCA1 (a) was used to treat macrophages. Si-control and Si-ABCA1 transfection reagent mixture was added into the medium and THP-1-derived macrophages were cultured at 37 °C for 5 h, then cells were treated with 50 μg/mL ox-LDL for 24 h. Further tests showed an increase in the degree of cell foaming and blocked cholesterol efflux (Fig. 3c, e, d). In addition, we also found that the transfected siABCA1 reagent was effective as shown by the inhibition of ABCA1 protein expression (Fig. 3g). And the expression of NLRP3 inflammasome and GSDMD (Fig. 3f, h, i) proteins was increased. At the same time, increased levels of LDH (Fig. 3k), mature IL-1β (Fig. 3j) in the medium and the increased proportion of positive cells after Hoechst33342/PI double staining also indicated that si-ABCA1 treatment can increase cell pyroptosis, which is characterized by cell pore formation (Fig. 3l). These results suggested that ABCA1-regulated blocking of cholesterol efflux led to increased cell pyroptosis in THP-1-derived macrophages.
Results indicate that ox-LDL-induced NLRP3-mediated cell signal activation was gradually increased with the increase in p-NFκB and p-IκB proteins, as a marker of NF-κB concentration-dependent manner. Additionally, the expression of NLRP3 inflammasome mediated macrophages pyroptosis in atherosclerosis [19]. In our study, we found that ox-LDL induced endothelial inflammation and delaying the development of AS lesions [15]. Zhuang et al. found in vivo that KLF2-FOXP13 transcriptional signal network can inhibit the expression of endothelial inflammasome NLRP3, alleviating endothelial inflammation and delaying the development of AS [19]. In our study, we found that ox-LDL induced NLRP3 inflammasome mediated macrophages pyroptosis in a concentration-dependent manner. Additionally, the expression of p-NFκB and p-IκB proteins, as a marker of NF-κB signal activation, was gradually increased with the increase of ox-LDL concentration. Therefore, the above experimental results indicate that ox-LDL-induced NLRP3-mediated cells pyroptosis may be associated with the activation of NF-κB signaling.

Activated NF-κB plays a pivotal role in the formation and stability of atherosclerotic plaques [20]. And in the presence of external risk factors such as high lipid induction, the key step in NF-κB activation is the phosphorylation of IκBα (I-Kappa-B-alpha) protein. Then p-IκBα is ubiquitinated and it releases NF-κB dimer, which is further activated by chemical modification such as phosphorylation, and then transported to the nucleus [21]. Liu et al. demonstrated that melatonin alleviates adipocyte pyroptosis by blocking NF-κB/GSDMD signaling in mouse adipose tissue [10], which suggests that NF-κB signaling may play an essential role in triggering pyroptosis. But it is not clear whether NF-κB activation has an effect on ox-LDL-induced THP-1 derived macrophage pyroptosis, which requires further study. In vitro, our experimental results indicated that inhibition of NF-κB signaling activation can effectively diminish ox-LDL-induced cell pyroptosis via inhibiting the activation of NLRP3 inflammasome, suggesting that NF-κB/NLRP3 pathway was required for ox-LDL induced macrophages pyroptosis, which is consistent with previous researches [7].

A recent study has shown that NF-κB activation may be associated with ABCA1 expression and cholesterol efflux in macrophages [22]. In agreement, we found that NF-κB signaling activation inhibitors significantly blocked ox-LDL inhibition of ABCA1 expression and increased cholesterol efflux in macrophage-derived foam cells, which suggests that under ox-LDL treatment, NF-κB signal acts as the upstream pathway of ABCA1 and is activated by ox-LDL to inhibit the expression of ABCA1, impeding the reverse cholesterol transport, and aggravating the degree of cell foaming.

A study in 2018 showed that cholesterol accumulation in bone marrow cells activate NLRP3 inflammasomes, further contributing to the development of AS plaques [15]. In myeloid ABCA1/G1-deficient LDLR−/− mice, the deletion of NLRP3 or caspase-1/11 reduced the size of atherosclerotic lesions [15]. In our study, we demonstrated for the first time in vitro that the inhibition of cholesterol reverse transport in macrophage-derived foam cells may increase the expression of NLRP3 inflammasome, thereby regulating cell pyroptosis and triggering inflammatory response.

Although some studies have shown that pyroptosis activates the inflammatory network and releases a large number of inflammatory factors, and thus accelerates the development of atherosclerosis, which is adverse to the development of AS and the stability of plaques [1, 2]. However, several studies have shown that cell pyroptosis plays an important role in controlling microbial infection. Costa Franco MMS et al. found that in the dendritic cells of C57BL/6 mice
Fig. 3 Dysfunction of ABCA1-regulated cholesterol efflux increases cell pyroptosis. 

- a, b The expression of ABCA1 protein was detected by Western Blot and the siABCA1 with the best inhibitory effect was selected. 
- c, d Oil red O staining (×40 magnification, the scale is 500 μm) and intracellular lipid titration detection in each of the treatment groups. 
- e Fluorescence reagent was used to detect the effluent capacity of cholesterol in foam cells after SiABCA1 treatment; 
- f-i western blot analysis of ABCA1, NLRP3 and GSDMD protein expression and gray scale of corresponding protein bands; 
- j ELISA was used to detect the content of IL-1β in the medium; 
- k lactate dehydrogenase release test; 
- l Hoechst33342/PI double staining was used to detect cell death; 

Among them, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, n = 3.
infected with Brucella, the genomic DNA of Brucella can cause pyroptosis of bone marrow-derived dendritic cells, which has been proved to be able to help control the infection and accelerate the clearance of the pathogen [23]. Wang et al. have recently shown that pyroptosis induced inflammation can trigger a powerful anti-tumor immune effect [24], which is conducive to tumor clearance. The above studies indicate that moderate pyroptosis, which is an important immune defense response of the body and plays an important role in resisting infection and endogenous danger signals, can quickly remove pathogens and risk factors, and enhance the immune function [25]. It was previously reported that treatment of smooth muscle cells with VX-765 at 50 μM effectively reduced pyroptosis [26]. Therefore, in this study, we treated macrophages with 50 μM VX-765, and found that ox-LDL-induced cell pyroptosis was significantly reduced. We also found for the first time that the accumulation of lipid droplets in foam cells was higher, and the intracellular cholesterol efflux was lower, suggesting that cell pyroptosis may be a double-edged sword in the development of AS.

In summary, NF-κB signaling pathway aggravation of ox-LDL-induced cell pyroptosis and cholesterol efflux disorder has been demonstrated in THP-1-derived macrophages for the first time, and this finding could lead to a novel therapeutic avenue for the treatment of AS.

Fig. 4 Cell apoptosis is a double-edged sword. Inhibition of cell apoptosis may increase the form. a After treatment, PI staining cell death was detected by flow cytometry; b, d oil red O staining (×40 magnification, the scale is 500 μm) and intracellular lipid titration detection in each of the treatment groups, c statistical analysis was made of the positive results of PI staining in each group in a. e Fluorescence test was used to detect the efflux capacity of cholesterol after treatment with pyroptosis inhibitors. *Represents control vs Ox-LDL; #stands for Ox-LDL vs OX-LDL+VX-765; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; #P in the same way, N = 3
Authors contributions JL and XG: conception, design and analysis of data, performed the data analyses, and wrote the manuscript. JL, JL and YY: contributed to the conception of the study. JL and YL: contributed significantly to analysis and manuscript preparation.

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Data availability My manuscript has no associated data.

Code availability None.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethical approval Not applicable.

Informed consent Not applicable.

Research involving human participants and/or animals Not applicable.

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