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**Original Paper**

# C/EBPβ Acts Upstream of NF-κB P65 Subunit in Ox-LDL-Induced IL-1β Production by Macrophages

Jun Ma[a] Chuan Liu[a] Yuanqi Yang[a] Jie Yu[a] Jie Yang[a] Sanjiu Yu[a] Jihang Zhang[a] Lan Huang[a]

[a]Institute of Cardiovascular Diseases, Xinqiao Hospital, Third Military Medical University, Chongqing, China

**Key Words**
C/EBPβ • NF-κB • IL-1β • Inflammation • Ox-LDL • Atherosclerosis

**Abstract**
***Background/Aims:*** Interleukin-1β (IL-1β) is one of the critical inflammatory factors during atherogenesis. CCAAT/enhancer binding proteins β (C/EBPβ), a regulator of IL-1β production, recently been evidenced as a key player in the development of atherosclerosis. However, the mechanisms of how C/EBPβ regulates the production of IL-1β are unclear. In this study, we aimed to explore the role of C/EBPβ in regulating IL-1β production in macrophages after oxidized low-density lipoprotein (ox-LDL) exposure and the underlying mechanisms. ***Methods:*** RAW264.7 macrophages were treated with 0, 25, 50 or 100 μg/ml ox-LDL for 12, 24 or 48 h. Small interfering RNAs were used to silence related proteins. The gene and protein expression levels were determined by quantitative real-time polymerase chain reaction or western blot (WB). IL-1β secretion was assessed by enzyme-linked immunosorbent assay. The cytoplasmic and nuclear proteins were evaluated by nuclear fractionation followed by WB. Localization of p65 was observed by immunofluorescence. The binding activity of p65 to IL-1β was tested by dual-luciferase reporter assay. ***Results:*** Ox-LDL increased IL-1β production, accompanied with increasing C/EBPβ and p65 expression in a dose- and time-dependent manner. Moreover, C/EBPβ deficiency in macrophages blocked ox-LDL-induced increases in IL-1β expression, maturation as well as p65 activation. However, p65 deficiency inhibited the increase in IL-1β production, but not C/EBPβ expression. Dual-luciferase reporter results showed that overexpression of C/EBPβ significantly enhanced binding activity of p65 to IL-1β promoter. In addition, C/EBPβ deficiency in macrophages abolished the ox-LDL-induced gene transcription increases of IL-1β, IL-6, p65 and caspase-1. ***Conclusions:*** Our results demonstrate that C/EBPβ acts upstream of NF-κB p65 subunit in ox-LDL-induced IL-1β production in macrophages and may regulate IL-1β maturation by promoting caspase-1. C/EBPβ may be a promising candidate for the prevention and treatment of atherosclerosis.

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Lan Huang and Jihang Zhang

Institute of Cardiovascular Diseases, Xinqiao Hospital, Third Military Medical University
Chongqing 400037 (China); Tel. 86-23-68755601, Fax 86-23-68755601,
E-Mail huanglan260@126.com, zhang.zjh@qq.com

## Introduction

Atherosclerosis, characterized by chronic inflammation of arteries, is the leading cause of cardiovascular diseases [1]. Macrophages play critical roles in atherogenesis. Macrophages engulf lipids, efflux cholesterol as high-density lipoprotein, and secrete a large number of inflammatory molecules, while macrophage apoptosis and death leads to unstable lesions that are more likely to rupture or fissure [2-5]. One of the earliest pathogenic events in atherosclerosis is the uptake of oxidized low-density lipoprotein (ox-LDL) by macrophages, which results in an inflammatory response and the formation of foam cells [6, 7]. Recent clinical evidence has shown that patients who receive long-term medication to control lipid metabolism dysfunction (such as statins), obtain significant clinical benefits. However, the residual risk of major adverse cardiovascular events in these patients is estimated to be 70–80%, possibly because current therapies do not address the inflammatory components of atherosclerosis [8]. Therefore, understanding the regulation of inflammation in macrophages is extremely important for the prevention and treatment of atherosclerosis.

Series of inflammatory factors secreted by macrophages play important roles in atherosclerosis [4, 9], including interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), monocyte chemotactic protein 1 (MCP-1), IL-6 and IL-10. Of these factors, IL-1β, one of the critical pro-inflammatory factors, has been shown to play key roles in the initiation, progression and rupture of atherosclerotic plaques [10-12]. Increased IL-1β serum level has been proved to be positively associated with the incidence and severity of coronary artery diseases [13]. In $apoE^{-/-}IL\text{-}1^{-/-}$ mice, the sizes of atherosclerotic lesions are significantly decreased compared with those in $apoE^{-/-}IL\text{-}1^{+/+}$ mice [14]. During atherogenesis, IL-1β activates platelets and induces vascular smooth muscle cells to produce other proinflammatory cytokines such as IL-6 and MCP-1 [15, 16]. A recent randomized controlled trial has demonstrated that canakinumab (a human IL-1β-targeted monoclonal antibody) significantly reduces recurrent cardiovascular events independent of lipid level, indicating a critical role of IL-1β in human atherogenesis. However, the use of canakinumab is associated with a higher incidence of fatal infection and sepsis [17]. Therefore, elucidation of the precise regulatory mechanisms underlying IL-1β production in macrophages is urgently needed for the prevention and treatment of inflammation in atherosclerosis.

Recently, the role of CCAAT/enhancer binding proteins β (C/EBPβ) in atherosclerosis has emerged. C/EBPβ is a basic Leucine Zipper (bZIP) transcription factor which binds to certain DNA regulatory regions to regulate gene expression [18]. Knockout of C/EBPβ gene in mice shows significant reduction of inflammatory cytokine levels and lesion areas in aortic sinuses compared with wildtype mice [19]. Generally, C/EBPβ was first found as a key regulator of IL-6 production [20]. Other studies have indicated that C/EBPβ may play important roles in the regulation of other inflammatory factors, such as IL-1β, MCP-1 and TNF-α [19]. However, the mechanisms by which C/EBPβ regulates inflammatory factors in macrophages, such as IL-1β production, in macrophages are still obscure. In addition, nuclear factor-kappa B (NF-κB), a protein complex composed of IκB bound to two proteins (p50 and p65), is the classical pathway in IL-1β production [21]. C/EBPβ has functional interaction with NF-κB p65 subunit, but the relationship of these two factors is still to be elucidated [22, 23].

Based on these considerations, we hypothesized that C/EBPβ may promote IL-1β production caused by ox-LDL through NF-κB pathway in macrophages.

## Materials and Methods

### *Cell culture and treatments*

RAW264.7 macrophage-like murine cells were obtained from the American Type Culture Collection (ATCC), cultured in RPMI-1640 (HyClone, Logan, UT, United States) supplemented with 7% fetal bovine serum (FBS, Gibco, Grand Island, NY, United States), 100 U/ml penicillin and 100 mg/ml streptomycin

(Beyotime, Shanghai, China) and kept in a humidified incubator (5% $CO_2$ in air) at 37°C. In our experiments, RAW264.7 cells were stimulated with 0, 25, 50 or 100 µg/ml ox-LDL (Yiyuan Biotechnologies, Guangzhou, China) for 12, 24 or 48 h, respectively. Cell supernatants were collected for enzyme-linked immunosorbent assays (ELISAs). Protein samples were used for western blot (WB) analysis, and RNA samples were used for quantitative real-time polymerase chain reaction (qPCR).

*Small interfering RNA transfection*

C/EBPβ, p65 and scrambled siRNAs were obtained from GenePharma Corp (Shanghai, China), and the sequences were as follows: C/EBPβ siRNA (sense, 5′-CCAUGGAAGUGGCCAACUUTT-3′ and antisense, 5′-AAGUUGGCCACUUC CAUGGTT-3′); p65 siRNA (sense, 5′-GGAGUACCCUGAAGCUAUATT -3′; antisense, 5′-UAUAGCUUCAGGGUACUCCTT-3′); scrambled siRNA (sense, 5′-UUCUCCGAACGUGUCACGUTT-3′; antisense, 5′-ACGUGACACGUUCG GAGAATT-3′). RAW264.7 cells were cultured in 6-well plates (Corning Life Sciences, Acton, MA, United States) for 12 h. Cells were then transfected with a mixture containing 7 µl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States) and 5 µl of siRNA in 500 µl of Opti-MEM (HyClone). After transfection, the cells were incubated for another 12 h, and the medium was then changed to RPMI-1640 containing the indicated concentrations of ox-LDL. Knockdown efficiency was determined by WB analysis.

*Enzyme-linked immunosorbent assay*

RAW264.7 cells were seeded in a 6-well plate (Corning) and treated as described above. IL-1β levels in the cell supernatants were measured using ELISA Quantikine kits (R&D Systems, Minneapolis, MN, United States) following the manufacturer's instructions.

*Quantitative real-time polymerase chain reaction*

Total RNA was isolated from treated RAW264.7 cells using RNAiso reagent (Takara, Ohtsu, Japan). Isolated RNA was reversely transcribed to cDNA using a PrimeScript RT reagent Kit (Takara) according to the manufacturer's protocols. Quantitative PCR was performed using a SYBR Premix Ex Taq II kit (Takara). All primers (Takara) were shown in Table 1. The GAPDH mRNA level was measured as an internal control.

*Western blot analysis*

Following treatments, RAW264.7 cells were collected and homogenized in lysis buffer (Beyotime) containing protease and phosphatase inhibitors (Roche Diagnostics Corp, Basel, Switzerland) and incubated on ice for 30 min. The whole cell lysates were then centrifuged at 15, 000 × g for 15 min. Protein concentrations were determined by BCA assay (Beyotime). The proteins in nucleus and cytoplasm were extracted by Nuclear and Cytoplasmic Protein Extraction kit (Beyotime) following manufacturer's instructions. The whole cell lysates were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore Corp, Billerica, MA, United States). The membranes were blocked with 5% non-fat milk in Tris-buffered saline solution with the detergent Tween-20 (TBST) at room temperature for 1 h and subsequently incubated with specific primary antibodies against IL-1β (Abcam, Cambridge, MA, USA, 1:1000), C/EBPβ (Abcam, 1:1000), GAPDH (Abcam, 1:2000), p65 (Cell Signaling Technology, Beverly, MA, United States, 1:1000) and phospho-p65 (Cell Signaling Technology, 1:1000) at 4°C overnight. The membranes were washed with TBST for three times and then incubated with an HRP-conjugated secondary antibody (Abcam, 1:2000) at 37°C for 1 h. The bands of protein were visualized by chemiluminescence detection and quantified by Image Quant TL software (GE Healthcare, Sweden).

**Table 1.** Primers and probes used in the study

| Gene(mouse) | primers |
|---|---|
| C/EBPβ | Forward: 5’ -GTTTCGGGACTTGATGCAAT-3’<br>Reverse: 5’ -CCCGCAGGAACATCTTTAAG-3’ |
| p65/NF-κB | Forward: 5’ -TGGTTCTGGTGGTGGTTACA-3’<br>Reverse: 5’ - GTCCCGGCATTATTAGCAGA-3’ |
| IL-1β | Forward: 5’ - GCTGCTTCCAAACCTTTGAC-3’<br>Reverse: 5’ - AGCTTCTCCACAGCCACAAT-3’ |
| IL-6 | Forward: 5’ - CCTCTGGCGGAGCTATTGAG-3’<br>Reverse: 5’ - CGGCAAGTGAGCAGATAGCA-3’ |
| IL-10 | Forward: 5’ - GGAGGTGCTGCTTGTGACAG-3’<br>Reverse: 5’ - TTGACTGCTGGCGATATGCT-3’ |
| NLRP3 | Forward: 5’ - GGAGGTGCTGCTTGTGACAG-3’<br>Reverse: 5’ - TGCCACCTTCTGACCAGTGT-3’ |
| Caspase-1 | Forward: 5’ - TCACCTTCAGCTCCAGACTCC-3’<br>Reverse: 5’ - TGGTGAGACTGAGGCCAATG-3’ |
| IKB-α | Forward: 5’ - AGCTGTCCTTGGCAGTCTCC-3’<br>Reverse: 5’ -TCTGACTGTTGACGCTGCTG-3’ |
| CD36 | Forward: 5’ - AATGTTGGTCCTGCCAATCC-3’<br>Reverse: 5’ -TCTCGGCGTGACTCTAACGA-3’ |
| TLR4 | Forward: 5’ - GCATGGCTTACACCACCTCTC-3’<br>Reverse: 5’ -TGTCTCCACAGCCACCAGAT-3’ |
| GAPDH | Forward: 5’ - ATCCTGTAGGCCAGGTGATG-3’<br>Reverse: 5’ -TATGCCCGAGGACAATAAGG-3’ |

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*Immunofluorescence*

RAW264.7 cells were seeded in 24-well plates with poly-L-lysine-coated coverslips at a density of 2×105 cells/ml. After treatment, the coverslips were washed with PBS three times, treated with 4% paraformaldehyde for 15 min at room temperature, and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, United States). After washed, the slides were incubated with a primary antibody against p65 (Cell Signaling Technology, 1:300) at 4°C overnight then incubated with Cy3-conjugated goat anti-rabbit IgG (Beyotime) for 1 h at room temperature. DAPI solution was added to the slides for 5 min. The slides were sealed with an anti-fluorescence quenching sealant (Beyotime) and observed using a Nikon C2 confocal laser scanning microscope.

*Luciferase reporter assay*

For the promoter of IL-1β luciferase reporter assays, the Coding Sequence (CDS) for NF-κB p65 subunit was synthesized and inserted into pcDNA3.1-EGFP vectors (Invitrogen) and CDS for protein of C/EBPβ was synthesized and inserted into pcDNA3.1 (Invitrogen), the IL-1β promoter (specific binding region of p65 to IL-1β) was synthesized and inserted into the pGL3-promoter vectors (Promega). The pGL3-promoter-IL-1β vectors and pcDNA3.1-p65 or its negative control along with pcDNA3.1 vectors or pcDNA3.1 -C/EBPβ vectors (Invitrogen) were co-transfected into RSC96 cells using Lipofectamine 2000. The pGL3-control vector was used as an internal control. Luciferase activity was measured after 72 h by the Dual Luciferase Reporter Assay Kit (Promega) according to manufacturer's instructions. All the recombinant vectors were validated by sequencing method (data not shown). The synthetic sequences were provided by Wuhan Gene Create Biological Engineering Co., Ltd, China.

*Statistics*

Statistical analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, United States), and groups were compared using one-way analysis of variance (ANOVA) followed by Tukey's studentized range (HSD) test. The data are presented as mean ± standard error of mean (SEM) of three independent experiments. P<0.05 was considered statistically significant.

## Results

*Ox-LDL increased IL-1β production in a dose- and time-dependent manner in macrophages*

Cells treated with ox-LDL at 25 μg/ml for 12 h significantly increased IL-1β mRNA expression compared with control and showed dose-dependent increase at 50 or 100 μg/ml of ox-LDL (Fig. 1A). Similarly, macrophages treated with different concentrations of ox-LDL for 24 h or 48 h also showed dose-dependent IL-1β mRNA increases (Fig. 1A). Furthermore, similar to the changes observed in gene expression, ox-LDL also significantly increased IL-

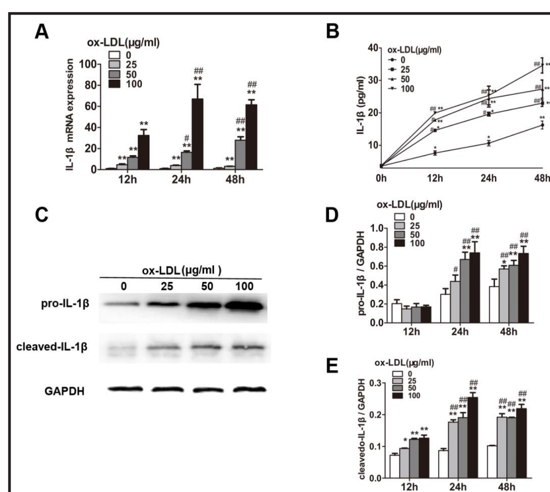


**Fig. 1.** Ox-LDL time- and dose-dependently increased IL-1β expression and secretion in macrophages. Cells were treated with 0, 25, 50 or 100 μg/ml ox-LDL for 12, 24 or 48 h. (A) IL-1β mRNA expression was analyzed by qPCR. (B) IL-1β secretion was analyzed via ELISA. (C) WB analysis of macrophages treated with ox-LDL at concentrations of 0, 25, 50 or 100 μg/ml for 24 h. (representative image) (D, E) Quantifications of pro-IL-1β and cleaved-IL-1β protein levels. (n=3 for each test; mean ± SEM; *P<0.05 and **P<0.01 vs 0 μg/ml at the same time points; #P<0.05, ##P<0.01 vs 12 h within same concentration).

1β secretion in a dose- and time-dependent manner (Fig. 1B). At the protein level, results showed that ox-LDL, starting at 50 μg/ml for 24 h or longer time, significantly increased pro-IL-1β expression (Fig. 1C, D). Notably, cleaved-IL-1β (maturation of IL-1β) expression was significantly increased by ox-LDL starting at 25 μg/ml for 12 h (Fig. 1E).

*C/EBPβ was required in IL-1β production in macrophages following ox-LDL exposure*

Ox-LDL at 50 μg/ml for 12 h in macrophages significantly increased C/EBPβ gene expression and showed a dose- and time-dependent manner with time and concentration increase (Fig. 2A). Besides, WB showed that ox-LDL dose- and time-dependently increased

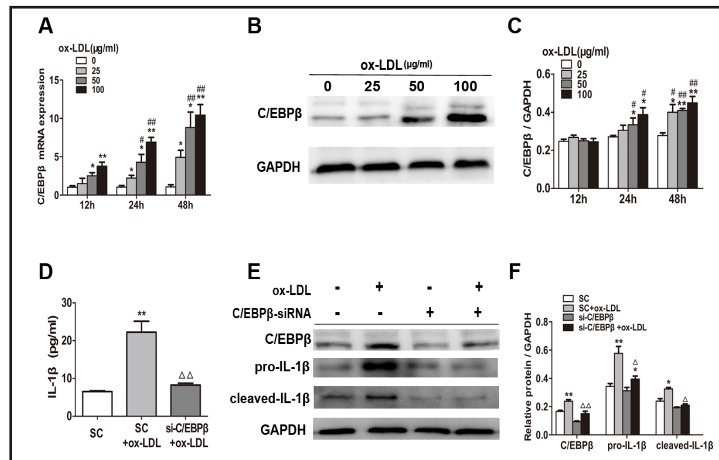

**Fig. 2.** C/EBPβ was involved with IL-1β expression and maturation in macrophages exposed to ox-LDL. Cells were exposed to ox-LDL at different concentrations (0, 25, 50 or 100 μg/ml) for 12, 24 or 48 h. (A) qPCR was used to measure C/EBPβ gene expression. (B) WB analysis of macrophages treated with ox-LDL at concentrations of 25, 50 or 100 μg/ml for 24 h. (representative image). (C) Quantifications of C/EBPβ protein expression. (D) C/EBPβ expression was knocked down by siRNA (C/EBPβ-siRNA), and the cells were then stimulated with 50 μg/ml ox-LDL for 24 h. IL-1β secretion was measured by ELISA. (E) After C/EBPβ was knocked down, WB analysis of macrophages treated with ox-LDL at concentrations of 50 μg/ml for 24 h. (representative image). (F) Quantitative of cleaved-IL-1β, pro-IL-1β and C/EBPβ protein levels. (n=3 for each test; mean ± SEM; scrambled siRNA, SC, was used as a negative siRNA control; *P<0.05 and **P<0.01 vs 0 μg/ml at the same time points or scrambled siRNA; #P<0.05, ##P<0.01 vs 12 h within same concentration; △P<0.05 and △△P<0.01 vs SC + ox-LDL).

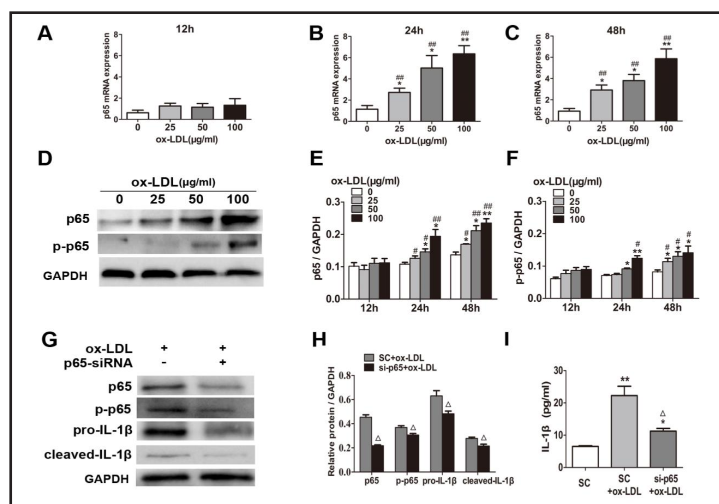

**Fig. 3.** p65 was required for IL-1β expression and maturation in macrophages. Cells were treated with 0, 25, 50 or 100 μg/ml ox-LDL for 12, 24 or 48 h. (A-C) p65 mRNA expression was analyzed by qPCR. (D) WB analysis of macrophages following treatment with 25, 50 or 100 μg/ml ox-LDL for 24 h (representative image). (E-F) Quantifications of p65 protein expression and phosphorylation. (G) After p65 was knockdown, WB analysis of macrophages treated with ox-LDL at concentrations of 50 μg/ml for 24 h (representative image). (H) Quantifications of cleaved-IL-1β, pro-IL-1β and p65 protein expression and p65 phosphorylation detected by WB. (I) IL-1β secretion was analyzed by ELISA. (n=3 for each test; mean ± SEM; scrambled siRNA, SC, was used as a negative siRNA control; *P<0.05 and **P<0.01 vs 0 μg/ml at the same time points or SC; #P<0.05, ##P<0.01 vs 12 h within same concentration; △P<0.05 and △△P<0.01 vs SC + ox-LDL).

C/EBPβ protein expression (Fig. 2B, C). Intriguingly, C/EBPβ-targeted siRNA abolished ox-LDL-induced IL-1β secretion (Fig. 2D). At the protein level, the increases of pro-IL-1β and cleaved-IL-1β by ox-LDL treatment were blocked by C/EBPβ knock-down, whereas siRNA-C/EBPβ alone had no effect on either pro-IL-1β or cleaved-IL-1β expression (Fig. 2E, F).

*p65 was involved in ox-LDL-induced IL-1β production*

Our data showed that starting at 25 μg/ml ox-LDL for 24 h, ox-LDL significantly increased p65 mRNA level compared with that in control cells, and p65 was further increased with 50 or 100 μg/ml ox-LDL for 24 or 48 h (Fig. 3A, B, C). Similarly, the protein expressions of p65 as well as phosphorylation (p-p65) status were also enhanced with ox-LDL concentration and time increase (Fig. 3D, E, F). Knocking down of p65 decreased p65 protein and phosphorylation but also inhibited pro-IL-1β and cleaved-IL-1β expression (Fig. 3G, H) as well as IL-1β secretion induced by ox-LDL (Fig. 3I).

*C/EBPβ regulated p65 but not vice versa*

To confirm the relationship of C/EBPβ and p65 in macrophages, we detected the protein expression using siRNA technique. WB results showed that C/EBPβ deficiency in macrophages inhibited ox-LDL-induced increases of p65 protein expression and phosphorylation in total proteins, whereas p65 deficiency did not affect the expression of C/EBPβ (Fig. 4A-D). Furthermore, ox-LDL increased the expression of C/EBPβ in both nucleus and cytoplasm but up-regulated the expression of p-p65 in nucleus only. C/EBPβ deficiency decreased p-p65 expression in the nucleus after ox-LDL treatment whereas knocking down p65 had no effect on C/EBPβ (Fig. 4E-G). All of these results suggested that C/EBPβ regulated p65, whereas p65 had no effect on C/EBPβ. To investigate the role of C/EBPβ on p65 function, we used dual luciferase reporter assay to test the p65-IL-β binding activity. Data showed that overexpression of C/EBPβ plus p65 significantly enhanced binding activity of p65 to

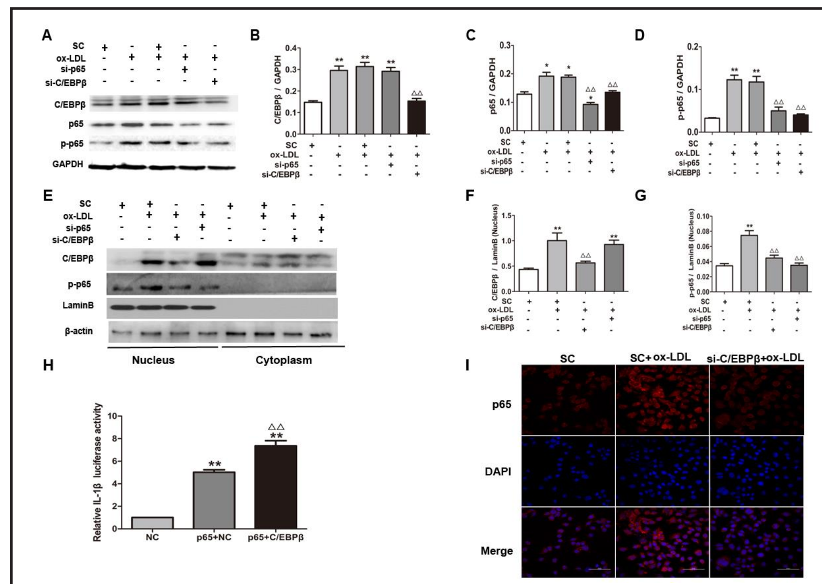


**Fig. 4.** Relationship of C/EBPβ and p65 in IL-1β production. (A) Cells were treated with 50 μg/ml ox-LDL for 24 h after C/EBPβ or p65 knocking down then the total protein of cells was detected by WB (representative image). (B-D) Quantifications of C/EBPβ and p65 protein expression and p65 phosphorylation in total protein. (E) Cells were treated with 50 μg/ml ox-LDL treatment for 24 h following C/EBPβ or p65 knock-down. The proteins in the nucleus and cytoplasm were detected by cell fractionation followed by WB(representative image). (F-G) Quantifications of C/EBPβ and p-p65 expressions in the nucleus and cytoplasm. (H) Following p65 or p65 plus C/EBPβ overexpression, the binding activity of p65 to IL-1β promoter was detected by dual luciferase reporter assay. (I) Immunofluorescence was used to analyze the localization p65 within cells after C/EBPβ knockdown. (n=3 for each test; mean ± SEM; scrambled siRNA, SC, was used as negative siRNA control; pCDNA3.1+IL-1beta was used as negative siRNA control, NC; *P<0.05 and **P<0.01 vs SC or NC; △P<0.05 and △△P<0.01 vs SC + ox-LDL or p65+NC).

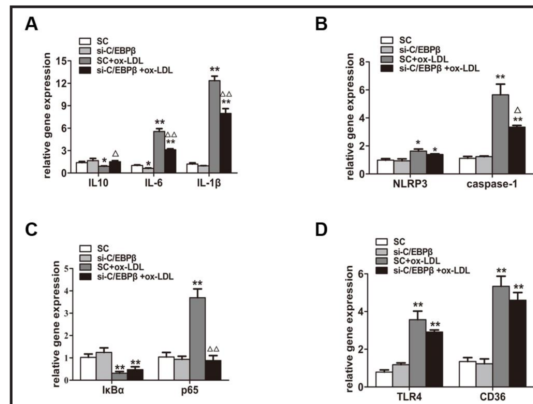

**Fig. 5.** Roles of C/EBPβ in ox-LDL-induced compensatory pathways that regulate IL-1β production. Cells were treated with ox-LDL 50 ug/ml for 24 h following siRNA-C/EBPβ, different gene mRNA levels were detected by qPCR. (A) IL-10, IL-6, and IL-1β; (B) NLRP3 and caspase-1; (C) IκBα and p65; (D) TLR4 and CD36 (n=3 for each test; mean ± SEM; scrambled siRNA, SC, was used as negative siRNA control; *P<0.05 and **P<0.01 vs SC, ΔP<0.05 and ΔΔP<0.01 vs SC + ox-LDL).

IL-1β promoter compared with p65 alone (Fig. 4H). Additionally, immunofluorescence revealed that ox-LDL increased the immunofluorescent intensity of p65 in both cytoplasm and nucleus, whereas this intensity increase was abolished by silencing C/EBPβ (Fig. 4I).

*C/EBPβ was involved in ox-LDL-induced compensatory pathways that regulate IL-1β production*

To investigate the role of C/EBPβ in IL-1β regulation induced by ox-LDL in macrophages, we detected a series of mediators, which are closely associated with IL-1β, by qPCR upon C/EBPβ knockdown. IL-6 and IL-10 are also important inflammatory factors in atherosclerosis [24, 25]. Results showed ox-LDL enhanced not only IL-1β but also increased IL-6 and decreased IL-10 gene transcription. Nevertheless, C/EBPβ deficiency attenuated the increases in IL-6 and IL-1β gene expression and the decrease in IL-10 expression induced by ox-LDL (Fig. 5A). NLR family pyrin domain-containing 3 (NLRP3) and caspase-1 gene expression, which are the major regulators to promote IL-1β maturation [26] and cytokine secretion in macrophages [27, 28], were significantly increased following ox-LDL treatment. However, C/EBPβ deficiency in macrophages blocked the ox-LDL-induced increase in caspase-1, but not NLRP3 (Fig. 5B). Additionally, ox-LDL decreased IκBα expression, the p65 activation inhibitor [29], and increased p65, while C/EBPβ deficiency blocked the ox-LDL-induced increase in p65, but did not reverse the decrease in IκBα mRNA level induced by ox-LDL (Fig. 5C). CD36 and TLR4, the important ox-LDL-associated receptors in macrophages, are believed to be involved in IL-1β production [30]. Our data showed that ox-LDL exposure increased the expression of CD36 and TLR4, but C/EBPβ deficiency had no effect on their gene expression (Fig. 5D).

## Discussion

In summary, we found that ox-LDL exposure increased IL-1β production, as well as C/EBPβ and p65 expression, in a dose- and time-dependent manner. Importantly, C/EBPβ deficiency abolished ox-LDL-induced increase in IL-1β production and p65 activation, whereas silencing of p65 inhibited the increase in IL-1β production but not C/EBPβ expression. Additionally, C/EBPβ deficiency blocked ox-LDL-induced caspase-1 elevation. These results suggest that C/EBPβ, acting upstream of p65, is essential for ox-LDL-induced IL-1β production, and may regulate IL-1β maturation by promoting caspase-1 expression.

IL-1β might be the primary inflammatory factor in ox-LDL-induced inflammation in macrophages. Our results and others [31-33] showed that ox-LDL also increased the IL-6 and decreased IL-10. However, IL-1β was more obviously affected by ox-LDL than IL-6 and IL-10 in our experiments. As the classical regulator of IL-1β, p65 regulates gene transcription by translocating into the nucleus from the cytoplasm and binding to its target DNA sequences [34]. Our results also indicated that p65 was activated by ox-LDL, and p65 activation was

required for IL-1β production. In addition to IL-1β gene and protein expressions, ox-LDL also promoted its maturation (showed by cleaved IL-1β), which is similar to previous results [35, 36]. Caspase-1 is the major regulator that promote IL-1β maturation and is mainly regulated by NLRP3[37]. We showed that ox-LDL increased caspase-1 and NLRP3 gene expression, implicating their participation in IL-1β maturation. Taken together, IL-1β expression via p65 and IL-1β maturation through caspase-1 may both contribute to ox-LDL-induced IL-1β production.

Moreover, we showed that ox-LDL increased C/EBPβ expression in macrophages, but the mechanisms are unclear. It has been shown that TLR4, a key receptor in macrophages, directly activates C/EBPβ [38]. Our results indicated that ox-LDL exposure also increased TLR4 gene expression, indicating that ox-LDL may up-regulate C/EBPβ through TLR4, but the detailed mechanism requires further study.

The involvement of C/EBPβ in IL-1β expression has been found not only by our study but also by others [39], but the mechanisms have not been clearly elaborated. Although it has been shown that there is C/EBP family binding site in the promoter region of the IL-1β gene, implying the possibility of direct binding [40], there is no further evidence supporting the direct regulation of C/EBPβ on IL-1β gene. On the contrary, we showed that C/EBPβ indirectly regulated IL-1β production through p65/NF–κB pathway.

Pervious study showed possible cooperativity between C/EBPβ and NF-κB in Interleukin-8 regulation in HeLa cells [41], but our evidence strongly suggest that C/EBPβ is the upstream regulator of p65 in IL-1β production, as silencing C/EBPβ decreased p65 expression and phosphorylation whereas silencing p65 had no effects on C/EBPβ in macrophages. (Fig. 4A-E). The difference might be attributed to different target gene or different treatment (ox-LDL vs TNF-α). Furthermore, C/EBPβ-upregulated p65 is likely due to mechanisms other than direct transcriptional activation, as no direct C/EBPβ binding site in the p65 gene has been found [22, 40]. IκBα may be one of the possible candidates that are upstream of p65 as previous study showed that C/EBPβ enhanced NF–κB-associated signaling by reducing the level of IκBα [42]. However, our results showed that although there is a trend, C/EBPβ deficiency did not significantly reverse the decrease of IκBα by ox-LDL, not supporting the involvement of IκBα in C/EBPβ–mediated p65 activation. This difference may due to different stimulators (LPS vs ox-LDL) and different cell lines. Moreover, CD36, another important upstream regulator of p65 12, may not be involved in C/EBPβ-mediated p65 activation, as in our study, C/EBPβ deficiency did not attenuated CD36 increase by ox-LDL. Additional studies are required for elucidating further detailed mechanisms. Besides, Phosphorylated p65 has been accepted as active form of p65 [43]. Our results showed that ox-LDL increased C/EBPβ accompanied with the increased of p65 phosphorylation as well as p65 expression. Thus, we could not conclude whether C/EBPβ regulates p65 activity through phosphorylation of p65 as the protein level of p65 also changed with phosphorylated level. Additionally, our results demonstrated that C/EBPβ overexpression significantly enhanced the binding activity of p65 to IL-1β promoter (Fig. 4H), which were consistent with previous study that C/EBPβ inhibition reduces NF-κB-DNA binding activity [44].

*C/EBPβ may contribute to IL-1β maturation via caspase-1*

Our study suggested that ox-LDL increased cleaved-IL-1β protein expression at an earlier time point than that of pro-IL-1β, and the changes in cleaved-IL-1β expression were consistent with IL-1β secretion, suggesting that maturation of IL-1β contributes more to IL-1β secretion at earlier stage of ox-LDL stimulation. Moreover, following ox-LDL treatment, p65 deficiency partially inhibited the increase in IL-1β secretion, while C/EBPβ deficiency almost completely blocked the elevation in IL-1β secretion. These results indicated that there might be other compensatory pathways in addition to NF–κB that are involved in the C/EBPβ-mediated increase in IL-1β production following ox-LDL exposure, especially during IL-1β maturation. Our results showed that C/EBPβ deficiency in macrophages blocked the caspase-1 increase induced by ox-LDL (Fig. 5B), suggesting the possible contribution of caspase-1. However, C/EBPβ deficiency did not change NLRP3 expression. These findings

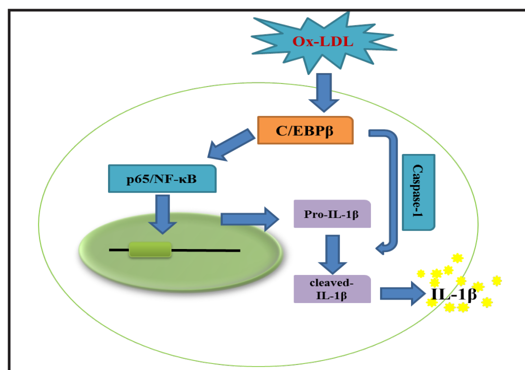


**Fig. 6.** Schematic drawing of how C/EBPβ regulates IL-1β production in macrophages. Ox-LDL promotes C/EBPβ in macrophages and C/EBPβ acts as an upstream molecule to mediate p65 activation, and then increases IL-1β expression in macrophages exposed to ox-LDL. C/EBPβ also promotes IL-1β maturation to affect IL-1β secretion possibly via caspase-1.

may be due to the involvement of other regulatory pathways, such as the non-NLR absent in melanoma 2 (AIM2) pathway, which also forms a caspase-1-containing inflammasome and promotes IL-1β maturation [45].

In our present study, we were not able to provide more evidence to elucidate the specific mechanisms of how ox-LDL increases the expression of C/EBPβ and by which C/EBPβ activates p65 and caspase-1. These are the limitations of our present study, which need to be further investigated.

In conclusion, we demonstrated that C/EBPβ, acting upstream of NF-κB p65 subunit, is essential for ox-LDL-induced IL-1β production, and promotes caspase-1 which may be involved in regulating IL-1β maturation (Fig. 6). Our findings may prompt subsequent studies and may provide new targets for treatment and prevention of atherosclerosis.

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## Disclosure Statement

The authors declare to have no conflict of interests.

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