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Original Article

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Macrophage-activating lipopeptide-2 downregulates the expression of ATP-binding cassette transporter A1 by activating the TLR2/NF-κB/ZNF202 pathway in THP-1 macrophages

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Abstract

Macrophage-activating lipopeptide-2 (MALP-2) has been shown to promote the development of atherosclerosis. ATP-binding cassette transporter A1 (ABCA1), a transmembrane protein, plays a critical role in mediating cholesterol export from macrophages to apolipoprotein A-I (apoA-I). However, whether MALP-2 can regulate the expression of ABCA1 is still largely unknown. The aim of this study was to explore the effects of MALP-2 on ABCA1 expression in THP-1 macrophages and the underlying mechanisms. Our results showed that the treatment of cells with MALP-2 decreased ABCA1 level and suppressed cholesterol efflux in both concentration- and time-dependent manners. The contents of intracellular cholesterol were significantly increased in the presence of MALP-2. Moreover, MALP-2-mediated inhibition of ABCA1 expression was abolished by siRNA of either Toll-like receptor 2 (TLR2) or nuclear factor κB (NF-κB). A similar effect was produced by treatment with the NF-κB inhibitor pyrrolidine dithiocarbamate. In addition, MALP-2-induced activation of NF-κB markedly increased zinc finger protein 202 (ZNF202) level, and ZNF202 siRNA impaired the effects of MALP-2 on ABCA1 expression. Taken together, these results suggest that MALP-2 can decrease ABCA1 expression and subsequent cholesterol efflux through activation of the TLR2/NF-κB/ZNF202 signaling pathway in THP-1 macrophages.

Key words: MALP-2, ABCA1, TLR2, NF-κB, ZNF202, cholesterol efflux

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Introduction

Atherosclerosis is the major etiology of multiple cardiovascular diseases including myocardial infarction and stroke, which are responsible for a large proportion of mortality in developed countries and increased prevalence in developing countries. Macrophage foam cell formation in the arterial intima has been considered as a critical event in the early stage of atherosclerotic lesions [1]. ATP-binding cassette transporter A1 (ABCA1), a transmembrane protein, mediates the transfer of intracellular cholesterol to apolipoprotein A-I (apoA-I) so as to form nascent high-density lipoprotein (HDL). ABCA1 thus plays a key role in both prevention of foam cell formation and promotion of reverse cholesterol transport (RCT) [2–4]. Mutations in human *ABCA1* gene cause familial HDL deficiency and Tangier disease, a rare lipid disorder characterized by very low level of HDL cholesterol, rapid catabolism of apoA-I, and high risk of developing coronary heart disease [5]. However, accumulating evidence indicates that overexpression of ABCA1 markedly enhances cholesterol efflux in macrophages [6] and protects against atherosclerosis in animal models [7]. Thus, it is important to further understand the mechanisms underlying ABCA1 expression so as to prevent and treat atherosclerotic diseases.

Toll-like receptors (TLRs) are critical pathogen recognition receptors in innate immunity. Among the members of the TLR family, TLR2 is highly expressed on the cell surface of the innate immune cells and can form a heterodimer with TLR6. Upon stimulation, TLR2 and TLR6 undergo conformational changes to recruit intracellular adapter molecules, such as myeloid differentiation primary response gene 88 (MyD88), leading to activation of nuclear factor κB (NF-κB) and subsequent upregulation of zinc finger protein 202 (ZNF202) expression [8,9]. It has been reported that macrophages from TLR2-null mice have a defect in foam cell formation in the presence of oxidized low-density lipoprotein (ox-LDL) [10], and that a global deficiency of TLR2 in LDL receptor (LDLr)-deficient mice fed a high-fat diet for 10 or 14 weeks is atheroprotective [11]. Recently, we found that *Chlamydia pneumoniae* negatively regulates ABCA1 expression and inhibits intracellular cholesterol release via the TLR2/NF-κB pathway in THP-1 macrophage-derived foam cells [12]. Another study from our laboratory showed that interleukin (IL)-18 in combination with IL-12 promotes nuclear translocation of NF-κB and then increases ZNF202 level, leading to downregulation of ABCA1 expression and subsequent foam cell formation in THP-1 macrophages [13]. These findings suggest that activation of the TLR2/NF-κB/ZNF202 signaling pathway is closely associated with the downregulation of ABCA1 expression.

Macrophage-activating lipopeptide-2 (MALP-2), a lipopeptide, was originally isolated from the cell wall of *Mycoplasma fermentans* [14], which can now be synthesized chemically. It functions as a potent agonist of TLR2. A recent study by Curtiss *et al.* [15] has demonstrated that treatment of LDLr-deficient mice fed a high-fat diet with MALP-2 remarkably accelerates atherosclerotic lesions in the abdominal segment of the descending aorta in a TLR2-dependent manner. Conversely, the administration of atorvastatin was found to decrease MALP-2 expression and then inhibit TLR2-mediated endothelial activation in human coronary artery endothelial cells [16]. However, it is unclear whether MALP-2 can regulate ABCA1 expression. To test this possibility, we explored the effects of MALP-2 on ABCA1 expression and the underlying molecular mechanisms in THP-1 macrophages. Our results show that exposure of cells to MALP-2 markedly reduces ABCA1 expression level and subsequent cholesterol efflux by activating the TLR2/NF-κB/ZNF202 pathway. These findings suggest that MALP-2 might be a potential target to suppress foam cell formation and progression of atherosclerosis.

Materials and Methods

Reagents and antibodies

MALP-2 was obtained from Alexis Biochemicals (Grünberg, Germany). RPMI 1640 medium and NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) were provided by Sigma (St Louis, USA). The rabbit monoclonal antibodies against ABCA1, TLR2, NF-κB p65, ZNF202, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). TRIzol reagent (Invitrogen, Carlsbad, USA), BCA protein assay reagent (Pierce Chemical, Rockford, USA), ReverAid™ first strand cDNA synthesis kit (Fermentas, Burlington, Canada), DyNAmoTM SYBR® Green qPCR Kits (Finnzymes, Espoo, Finland), and nitrocellulose membranes (Millipore, Boston, USA) were obtained as indicated, respectively. Additionally, ox-LDL was prepared as described previously [17].

Cell culture and treatment

Human THP-1 cells originally obtained from the American Type Culture Collection (ATCC, Manassas, USA) were seeded into six-well plates at 1.0×10^6 cells per well in RPMI 1640 medium supplemented with 0.1% nonessential amino acids, 20 U/ml penicillin, 20 μg/ml streptomycin, and 10% fetal bovine serum and then maintained in a humidified atmosphere of 5% CO_2 at 37°C. After 3–4 days, these cells were treated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) for 72 h and then exposed to 50 μg/ml ox-LDL for 48 h in a serum-free medium for full differentiation into macrophages. THP-1 macrophages were treated with different concentrations of MALP-2 (0, 25, 50, 100, and 200 mM) for 24 h or exposed to 100 mM MALP-2 for various durations (0, 6, 12, 24, and 48 h). Additionally, in some experiments, macrophages were incubated with or without PDTC (50 μM) or small interfering RNAs (siRNAs) for TLR2, NF-κB, and ZNF202 (Sangon Biotech, Shanghai, China) before MALP-2 stimulation.

Oil Red O staining

Cells were washed once with phosphate-buffered saline (PBS) and subsequently fixed with 4% paraformaldehyde for 10 min. After rinsing with 60% isopropanol, cells were stained with 0.3% Oil Red O in 60% isopropanol for 10 min and then washed with 60% isopropanol again, followed by counterstaining with hematoxylin for 3 min. After copious washing with water, cells were photographed using a microscope at 400× magnification.

Detection of intracellular cholesterol efflux

Cells were cultured as described above and then were incubated with 0.2 μCi/ml [^{3}H]cholesterol. After 72 h, cells were washed with PBS, followed by overnight incubation in RPMI 1640 medium containing 0.1% (w/v) bovine serum albumin (BSA) to allow equilibration of [^{3}H]cholesterol in all cellular pools. The cells were then washed with PBS and incubated in 2 ml of an efflux medium containing RPMI 1640 medium, 0.1% BSA, and human plasma apoA-I (25 μg/ml). A 150 μl sample of efflux medium was obtained at the indicated time and passed through a 0.45-μm filter to remove any floating cells. The cells were washed twice with PBS, and cellular lipids were extracted using isopropanol. Medium or cell-associated [^{3}H]cholesterol was then measured by liquid scintillation counting. Percent efflux was calculated by the following equation: [total media counts/(total cellular counts + total media counts)] × 100%.

High-performance liquid chromatography (HPLC) assay

HPLC analysis was performed as described in our previous publications [13]. Data were analyzed with Total Chrome software from PerkinElmer.

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Transfection of siRNA

THP-1 macrophages (2×10^6 cells/well) were transfected with non-silencing control siRNA, TLR2 siRNA, or NF-κB siRNA using Lipofectamine 2000 (Invitrogen) for 24 h according to the manufacturer's protocol.

RNA isolation and real-time quantitative PCR

Total RNA was extracted from cells using TRIzol reagent according to the manufacturer's instructions. The first strand cDNA was synthesized using ReverAid™ first strand cDNA synthesis Kit. Real-time quantitative PCR was conducted on a Roche Light Cycler Run 5.32 Real-Time PCR System (Roche, Basel, Switzerland) using SYBR Green detection chemistry. Melt curve analyses of all PCR products were performed and shown to produce a single DNA duplex. Quantitative measurements were determined by the ΔΔCt method and the expression of β-actin was used as the internal control.

Protein extraction and western blot analysis

Total cellular proteins for western blot detection of ABCA1, TLR2, and ZNF202 and nuclear proteins for the detection of NF-κB p65 were isolated from cells with various treatments using an NE-PER nuclear and cytoplasmic extraction reagents according to the manufacturer's instructions (Pierce, Rockford, USA). These protein extracts (20 μg of each sample) were subject to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were incubated with various primary antibodies and subsequent horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Santa Cruz Biotechnology), and finally visualized using a chemiluminescence method (ECL Plus Western Blotting Detection System; Amersham Biosciences, Foster City, USA). Densitometry was carried out using the Image J software (version 1.38).

Statistical analysis

Data are expressed as the mean ± SD. Results were analyzed by one-way ANOVA and Student's *t*-test, using the SPSS 13.0 software. Statistical significance was obtained when *P*-values were <0.05.

Results

MALP-2 decreases the expression of ABCA1 in THP-1 macrophages

To investigate the regulatory role of MALP-2 in ABCA1 expression, the mRNA and protein levels of ABCA1 were first examined in THP-1 macrophages treated with and without MALP-2 using real-time quantitative PCR and western blot assays. As shown in **Fig. 1A–D**, the

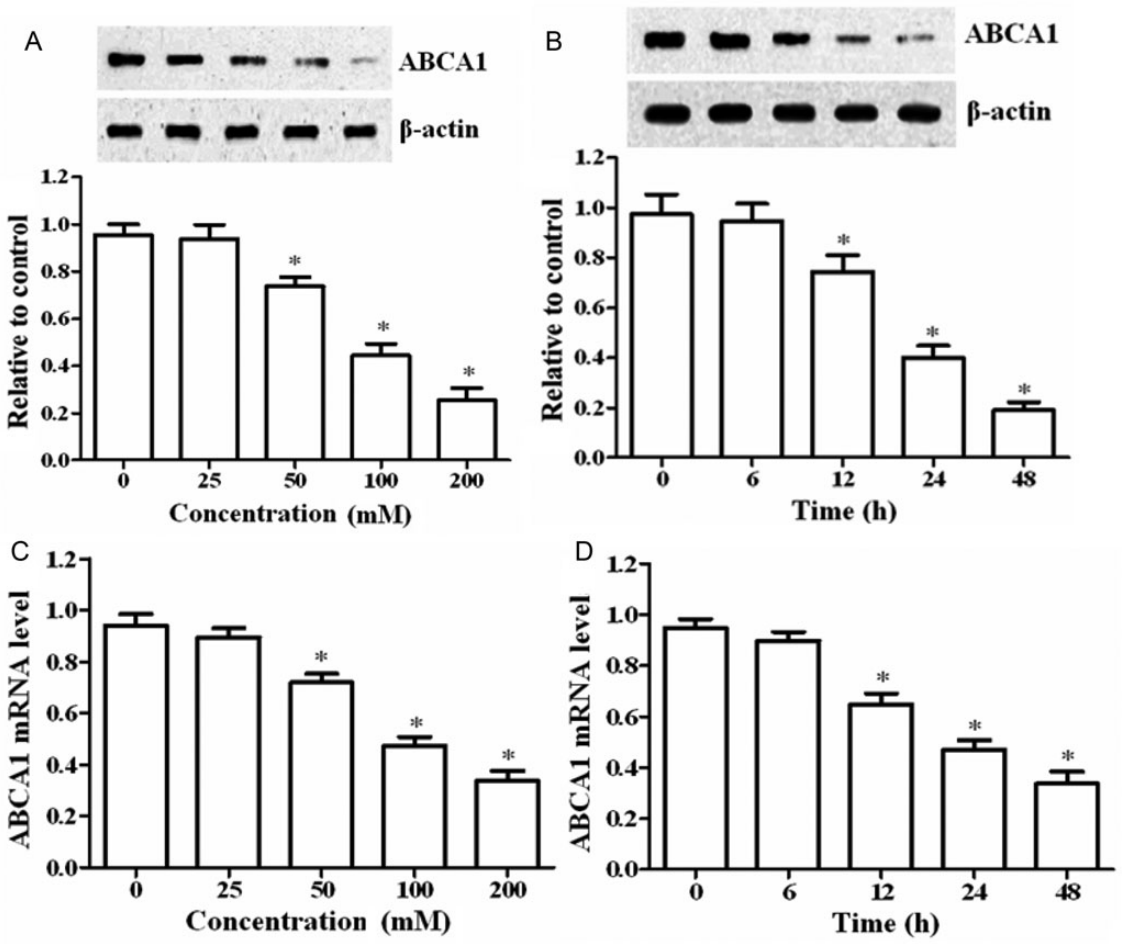

Figure 1. Effects of MALP-2 on the expression of ABCA1 in THP-1 macrophages (A–D) Cells were treated with indicated concentrations of MALP-2 (A,C) or incubated with MALP-2 (100 mM) for various time periods (B,D). (A,B) Protein samples were immunoblotted with anti-ABCA1 or anti-β-actin antibodies. (C,D) The expression of ABCA1 mRNA was determined by real-time quantitative PCR. Data are presented as the mean ± SD from three independent experiments, each in triplicate. *$P < 0.05$ vs. baseline.

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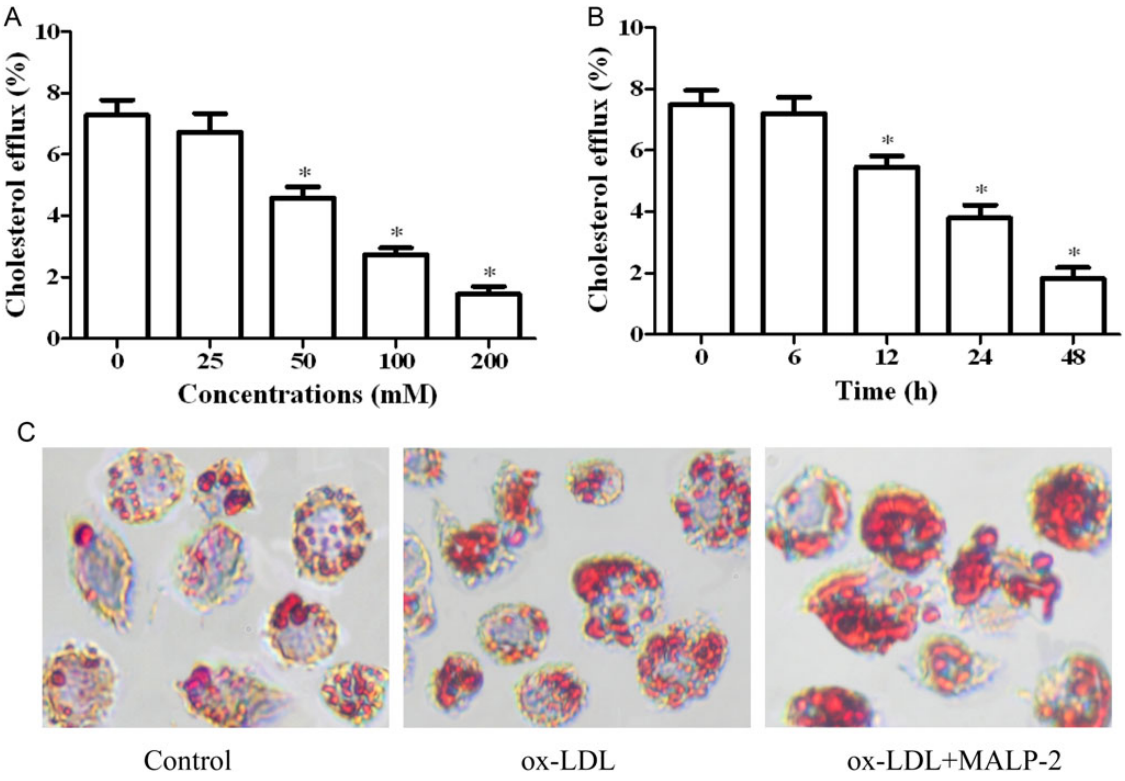

Figure 2. Effects of MALP-2 on cholesterol efflux and intracellular lipid accumulation in THP-1 macrophages (A,B) THP-1 macrophages were exposed to various concentrations of MALP-2 (A) or treated with 100 mM of MALP-2 for various time periods (B), and subsequently, cholesterol efflux was analyzed using liquid scintillation counting method. (C) THP-1 cells were incubated with or without ox-LDL and/or MALP-2 (100 mM) for 24 h, followed by Oil Red O staining. Data are presented as the mean ± SD of three independent experiments, each in triplicate. *$P < 0.05$ vs. baseline.

addition of MALP-2 significantly decreased the mRNA and protein levels of ABCA1 in both concentration- and time-dependent manners, suggesting that MALP-2 is a negative regulator of ABCA1 expression.

MALP-2 inhibits cholesterol efflux and promotes intracellular lipid accumulation

ABCA1 is known to play a critical role in promoting RCT and regulating intracellular cholesterol homeostasis by mediating cholesterol removal from macrophages to apoA-I. Given that MALP-2 downregulated ABCA1 expression, we further analyzed whether exposure of THP-1 macrophages to MALP-2 could affect cholesterol efflux and intracellular cholesterol contents. Our results showed that cholesterol efflux was significantly reduced when cells were treated with MALP-2 (**Fig. 2A,B**). In addition, treatment with MALP-2 markedly facilitated lipid accumulation and enhanced cholesterol contents within THP-1 macrophages, as revealed by Oil Red O staining (**Fig. 2C**) and HPLC (**Tables 1** and **2**), respectively. These findings suggest that MALP-2 increases the transformation of macrophages into foam cells possibly through suppression of ABCA1 expression.

TLR2/NF-κB pathway is involved in MALP-2-mediated downregulation of ABCA1 expression

Overexpression of TLR2 has been shown to decrease ABCA1 expression in THP-1 macrophage-derived foam cells [12]. Given that MALP-2 is an activator of TLR2, it is likely that TLR2 is involved in MALP-2-induced decrease in ABCA1 levels. To test this possibility, THP-1 macrophages were treated with TLR2 siRNA, followed by

Table 1. Concentration-dependent effects of MALP-2 on cholesterol contents in THP-1 macrophages

Concentration (mM)	TC (mg/g)	FC (mg/g)	CE (mg/g)	CE/TC (%)
0	418 ± 42	169 ± 21	249 ± 28	59.6
25	443 ± 46	175 ± 23	268 ± 32	60.5
50	483 ± 51*	190 ± 26*	293 ± 35*	60.7
100	528 ± 55*	206 ± 30*	322 ± 39*	61.0
200	579 ± 60*	223 ± 32*	356 ± 43*	61.5

THP-1 macrophages were treated with different concentrations of MALP-2 (0, 25, 50, 100, and 200 mM) for 24 h, respectively. HPLC was then performed to detect the levels of intracellular total cholesterol (TC), free cholesterol (FC), and cholesterol ester (CE). Data are presented as the mean ± SD from three independent experiments, each in triplicate.

*$P < 0.05$ vs. baseline.

treatment with and without MALP-2. As demonstrated in **Fig. 3A**, transfection with TLR2 siRNA inhibited the expression of TLR2 protein by 85%. Moreover, knockdown of TLR2 by siRNA partially reversed MALP-2-induced increase of NF-κB p65 (the transcriptionally active subunit of NF-κB) expression in the nuclei and MALP-2-induced downregulation of ABCA1 expression (**Fig. 3B,C**). Thus, these observations support an involvement of TLR2 in MALP-2-induced downregulation of ABCA1 expression.

It is well known that NF-κB is a downstream molecule of TLR2 [18]. Since the activation of NF-κB can downregulate ABCA1 expression [19], we speculated that NF-κB might also be involved in the

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effect of MALP-2 on ABCA1 expression. Indeed, the treatment of THP-1 macrophages with siRNA for NF-κB attenuated the levels of nuclear NF-κB p65 by 80% (**Fig. 3D**), and significantly reversed MALP-2-induced reduction of ABCA1 expression (**Fig. 3E,F**).

Table 2. Time-dependent effects of MALP-2 on cholesterol contents in THP-1 macrophages

Time (h)	TC (mg/g)	FC (mg/g)	CE (mg/g)	CE/TC (%)
0	439 ± 46	183 ± 22	256 ± 32	58.3
6	462 ± 49	191 ± 27	271 ± 35	58.6
12	507 ± 54*	205 ± 30*	302 ± 36*	59.6
24	546 ± 58*	217 ± 34*	329 ± 38*	60.3
48	625 ± 63*	238 ± 36*	387 ± 45*	61.9

THP-1 macrophages were exposed to 100 mM of MALP-2 for indicated time periods. HPLC was used to determine the contents of intracellular TC, FC, and CE. Data are presented as the mean ± SD from three independent experiments, each in triplicate.

*$P < 0.05$ vs. baseline.

To further validate this observation, THP-1 macrophages were incubated with PDTC, an inhibitor of NF-κB activity. A similar effect was found in response to PDTC treatment (**Fig. 3E,F**). Taken together, our data demonstrate that the inhibitory effects of MALP-2 on ABCA1 expression may result from the activation of the TLR2/NF-κB signaling pathway.

NF-κB-induced downregulation of ABCA1 expression is dependent on ZNF202

Accumulating evidence has demonstrated that ZNF202 is a transcriptional repressor of ABCA1 [20]. To define whether ZNF202 is associated with the effects of MALP-2, THP-1 macrophages incubated with MALP-2 were treated with or without NF-κB siRNA. Our results showed that MALP-2 obviously increased ZNF202 levels, which were blocked by NF-κB siRNA (**Fig. 4A**). We further treated cells with ZNF202 siRNA directly and found that siRNA for ZNF202 efficiently reduced ZNF202 expression by 83% (**Fig. 4B**) and significantly reversed MALP-2-induced suppression of ABCA1 expression (**Fig. 4C,D**). These findings suggest that ZNF202 plays a role in MALP-2-induced downregulation of ABCA1 expression.

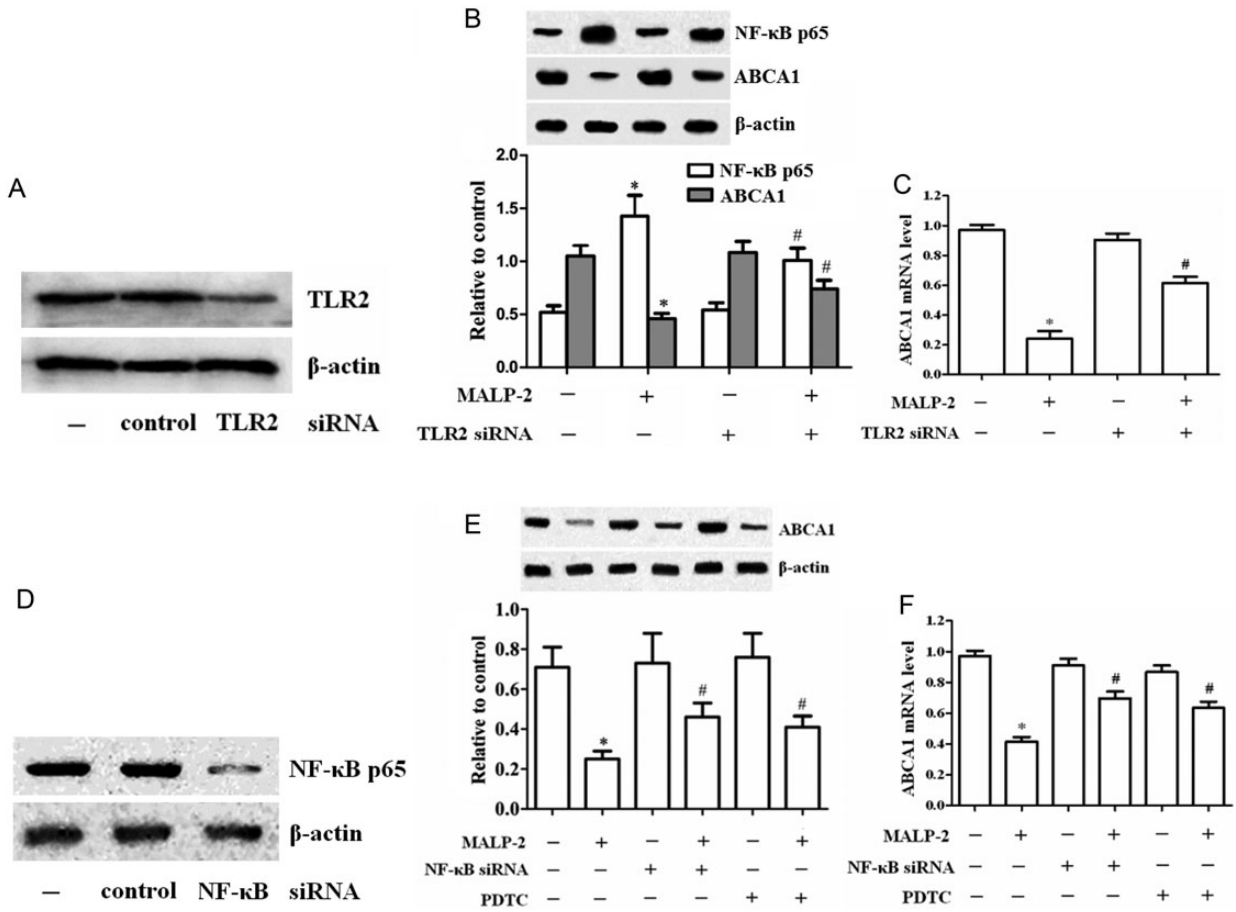

Figure 3. Involvement of the TLR2/NF-κB pathway in downregulation of ABCA1 expression induced by MALP-2 in THP-1 macrophages (A–C) Cells were transfected with control or TLR2 siRNAs for 24 h and then incubated with MALP-2 (100 mM) for another 24 h. (A,B) Western blot analysis was conducted to determine the expression of TLR2, ABCA1, and nuclear NF-κB p65. (C) The levels of ABCA1 mRNA were examined by real-time quantitative PCR. (D–F) Cells were pretreated with control siRNA, NF-κB siRNA, or PDTC (50 μM) for 24 h. These cells were then exposed to MALP-2 (100 mM) for another 24 h. (D,E) The nuclear and total cellular proteins were prepared, followed by western blot analysis of NF-κB p65 (D) and ABCA1 (E), respectively. (F) Real-time quantitative PCR was used to evaluate ABCA1 mRNA expression. Similar results were obtained in three independent experiments. Data are presented as the mean ± SD. *$P < 0.05$ vs. baseline; #$P < 0.05$ vs. MALP-2-treated group.

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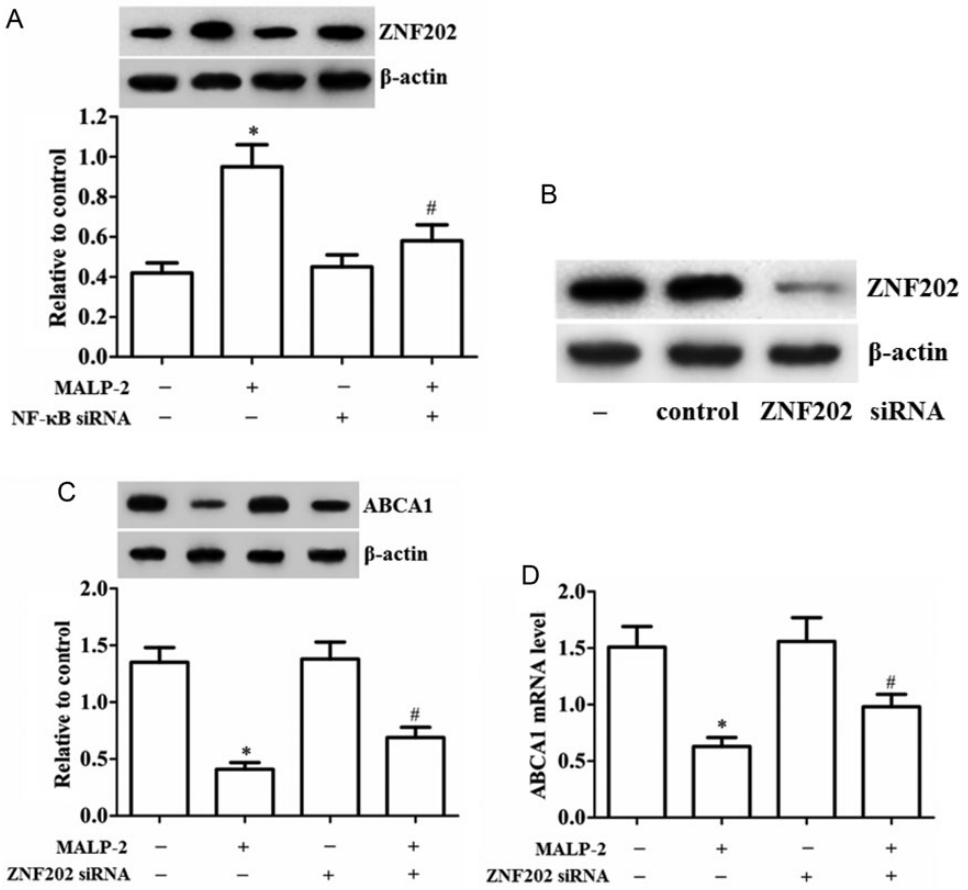

Figure 4. ZNF202 is implicated in MALP-2-mediated downregulation of ABCA1 expression in THP-1 macrophages (A) Cells were transfected with control or NF-κB siRNAs for 24 h, followed by incubation with MALP-2 (100 mM) for 24 h. The expression of ZNF202 was measured by western blot analysis. (B–D) Cells were treated with control or ZNF202 siRNAs for 24 h and then incubated with MALP-2 (100 mM) for another 24 h. (B) Protein samples were immunoblotted with anti-ZNF202 or anti-β-actin antibodies. (C,D) ABCA1 mRNA and protein were detected by real-time quantitative PCR and western blot analyses, respectively. Data are presented as the mean ± SD of three independent experiments, each in triplicate. $^{*}P < 0.05$ vs. baseline; $^{\#}P < 0.05$ vs. MALP-2-treated group.

Discussion

It is well established that maintaining intracellular cholesterol homeostasis is essential for normal human physiology. Formation of macrophage foam cells within the arterial wall results in the excessive internalization of lipoproteins, and subsequently promotes the development of early atherosclerotic plaques. ABCA1, a member of the large superfamily of ABC transporters, is known to play a critical role in the suppression of macrophage foam cell formation and atherogenesis through mediating the active transport of intracellular cholesterol and phospholipids to apoA-I, an extracellular acceptor [21]. These apoA-I-associated lipids are then delivered to the liver for excretion into the bile and eventually the feces. This process is called RCT. Although MALP-2 exerts a proatherogenic effect [15], there are no reports concerning its impacts on ABCA1 expression. Here, we revealed that exposure of THP-1 macrophages to MALP-2 reduced ABCA1 expression and intracellular cholesterol efflux in both concentration- and time-dependent manners. Moreover, intracellular lipids were significantly accumulated in the presence of MALP-2, suggesting that MALP-2 is a novel negative regulator of ABCA1 expression, which may partially explain its proatherogenic properties.

A total of 11 types of TLR have been identified in mammalian cells, among which TLR2 is the most intensively investigated one. In animal models of atherosclerosis, increased endothelial expression of TLR2 at the sites with blood flow disturbing has been shown to exacerbate early atherogenic events [22], whereas the loss of TLR2 leads to a marked decrease in atherosclerotic lesion size [11]. These findings highlight a close link between TLR2 and atherogenesis. In the present study, transfection of THP-1 macrophages with TLR2 siRNA dramatically blocked nuclear translocation of NF-κB and reversed the downregulation of ABCA1 expression induced by MALP-2, implicating a role for TLR2 in MALP-2-mediated activation of NF-κB and subsequent inhibition of ABCA1 expression. This finding is consistent with those in our previous report, in which an increase in NF-κB activity and a decrease in ABCA1 level induced by *C. pneumoniae* are mediated by TLR2 in THP-1 macrophage-derived foam cells [12]. However, a recent study by Park *et al.* [23] showed that treatment with Pam3CSK4, another agonist of TLR2, stimulates ABCA1 expression via triggering protein kinase C-η (PKC-η) and phospholipase D2 (PLD2) signaling pathway in a mouse macrophage cell line RAW264.7. This discrepancy may be attributed to the differences in the types of TLR2 agonists and the signaling pathways involved, but the role of TLR2 in the regulation of ABCA1 expression needs further research.

NF-κB is a well-known nuclear transcription factor. In resting condition, NF-κB exists in the cytoplasm in the form of homodimers or heterodimers and remains inactive because of its interaction with

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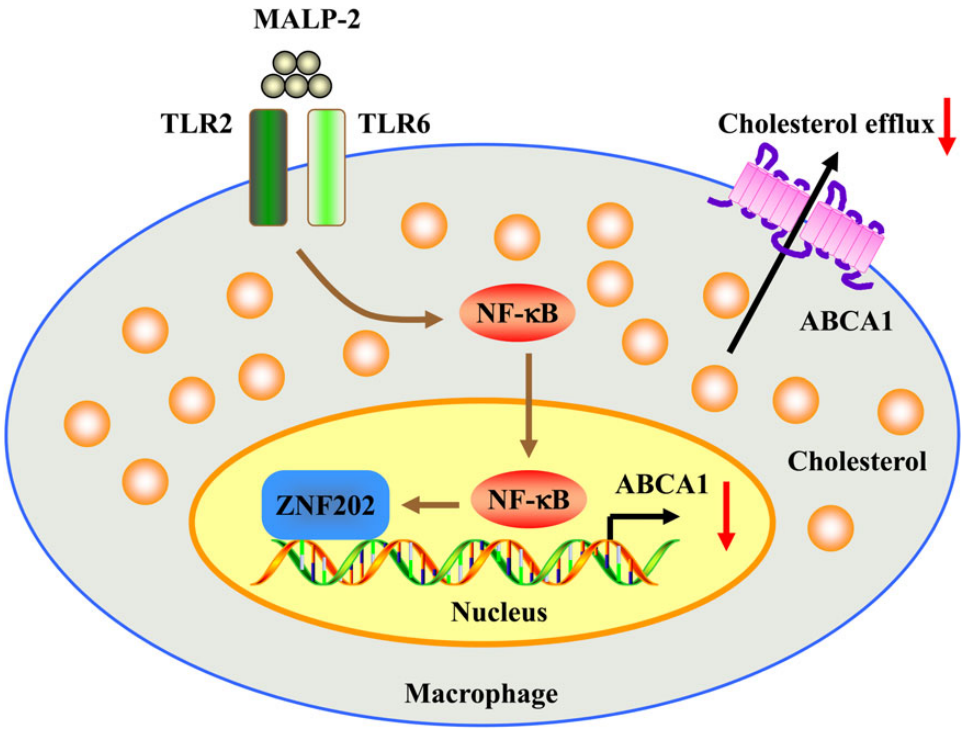

Figure 5. Schematic presentation of MALP-2-induced downregulation of ABCA1 expression Exposure of THP-1 macrophages to MALP-2 activates TLR2, leading to nuclear translocation of NF-κB. Activation of NF-κB then decreases ABCA1 levels and subsequent cholesterol efflux possibly by stimulating the expression of ZNF202, a transcriptional repressor of ABCA1.

inhibitor of κB (IκB). In response to extracellular stimuli, such as stress, cytokines, free radicals, and bacterial or viral antigens, NF-κB dissociates with IκB and translocates to the nucleus for transcription of target genes [24]. Many of these NF-κB activators and NF-κB-regulated genes are involved directly or indirectly in the atherosclerotic process [25]. Numerous studies from our group and others have revealed that activated NF-κB can suppress ABCA1 expression in several cell types, including THP-1 macrophage-derived foam cells [26], U937 cells [27], and RAW264.7 cells [28]. Here, we reported that MALP-2 significantly enhanced the levels of NF-κB p65 in the nuclei, indicating that MALP-2 can activate NF-κB. Moreover, MALP-2-induced downregulation of ABCA1 expression was significantly reversed when cells were exposed to NF-κB siRNA or its inhibitor PDTC, suggesting that NF-κB, as a downstream molecule of TLR2, is indeed involved in MALP-2 effects on ABCA1 expression.

Because the direct binding site of NF-κB on the ABCA1 promoter has not been identified, NF-κB may decrease ABCA1 expression indirectly through some intermediate molecules. ZNF202 is a transcriptional repressor of ABCA1 by binding to GnT repeats within the ABCA1 promoter [20]. Our previous studies have demonstrated that the activation of NF-κB inhibits ABCA1 expression by enhancing ZNF202 levels in THP-1 macrophage-derived foam cells [13]. In the current study, the treatment of cells with MALP-2 significantly increased the levels of ZNF202, which were partially reversed by NF-κB siRNA. Moreover, siRNA for ZNF202 effectively reduced MALP-2-mediated inhibition of ABCA1 expression. Thus, ZNF202 is also required for the effects of MALP-2 on ABCA1 expression.

In summary, our results have shown that MALP-2 exerts a significant inhibitory effect on ABCA1 expression in THP-1 macrophages, and the mechanisms may be associated with activation of the TLR2/NF-κB signaling pathway (**Fig. 5**). This study provides a novel insight into the proatherogenic actions of MALP-2. Blockade of MALP-2 might represent a promising therapeutic strategy to prevent foam cell formation and reduce the risks of atherosclerotic diseases.

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