

Original Paper

Curcumin Modulates Macrophage Polarization Through the Inhibition of the Toll-Like Receptor 4 Expression and its Signaling Pathways

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Key Words

Curcumin • Macrophage polarization • TLR4 • MAPK • NF-κB • Atherosclerosis

Abstract

Background: Curcumin, the active ingredient in curcuma rhizomes, has a wide range of therapeutic effects. However, its atheroprotective activity in human acute monocytic leukemia THP-1 cells remains unclear. We investigated the activity and molecular mechanism of action of curcumin in polarized macrophages. **Methods:** Phorbol myristate acetate (PMA)-treated THP-1 cells were differentiated to macrophages, which were further polarized to M1 cells by lipopolysaccharide (LPS; 1 μg/ml) and interferon (IFN)-γ (20 ng/ml) and treated with varying curcumin concentrations. [³H]thymidine (³H-TdR) incorporation assays were utilized to measure curcumin-induced growth inhibition. The expression of tumor necrosis factor-α (TNF-α), interleukin (IL-6), and IL-12B (p40) were measured by quantitative real-time polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). Macrophage polarization and its mechanism were evaluated by flow cytometry and western blot. Additionally, toll-like receptor 4 (TLR4) small interfering RNA and mitogen-activated protein kinase (MAPK) inhibitors were used to further confirm the molecular mechanism of curcumin on macrophage polarization. **Results:** Curcumin dose-dependently inhibited M1 macrophage polarization and the production of TNF-α, IL-6, and IL-12B (p40). It also decreased TLR4 expression, which regulates M1 macrophage polarization. Furthermore, curcumin significantly inhibited the phosphorylation of ERK, JNK, p38, and nuclear factor (NF)-κB. In contrast, siTLR4 in combination with p-JNK, p-ERK, and p-p38 inhibition reduced the effect of curcumin on polarization. **Conclusions:** Curcumin can modulate macrophage polarization through TLR4-mediated signaling pathway inhibition, indicating that its effect on macrophage polarization is related to its anti-inflammatory and atheroprotective effects. Our data suggest that curcumin could be used as a therapeutic agent in atherosclerosis.

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Introduction

Coronary artery disease (CAD), stroke, and other atherosclerosis-related diseases are considered the greatest health risk worldwide. Atherosclerosis is recognized as a chronic inflammatory disease. Monocyte-derived macrophages, which contribute to the immune and inflammatory response by scavenging and presenting antigens, play a key role in the initiation and progression of atherosclerosis. Macrophages are phenotypically heterogeneous, bearing distinct characteristics and functions [1]. Selective gene expression through different signaling pathways can induce the differentiation of M1- and M2-type macrophages. During the progression of atherosclerosis, M1 and M2 macrophage populations display differing location preferences on the vessel wall. Pro-atherogenic M1 cells dominate plaque shoulders, which are relatively vulnerable and rupture-prone, but are seldom found in fibrous cap regions. This suggests that the deleterious effects of M1 macrophages are countered by the reparative, atheroprotective effects of M2 macrophages. The plasticity of these subsets may exert a considerable influence on the outcome of the atherosclerosis. Excessive M1 activation may preferentially foster sustained inflammation and plaque progression [2]. In the atherosclerotic plaques of apoE knockout (KO) mice, the switch from an M2 profile in early atherosclerotic lesions to M1 in advanced plaques is consistent with the observation that polarized macrophages were capable of changing phenotype *in vitro* [3]. However, phenotypic switching between these two subsets in plaques has not been demonstrated *in vivo* [1]. Thus, macrophage heterogeneity could be a potential biomarker for atherosclerosis and therapeutic effect following drug treatment.

Curcumin is the active ingredient in curcuma rhizomes. Extensive animal research and clinical trials have shown that curcumin is a pleiotropic and pharmacologically safe polyphenol with a wide range of therapeutic effects [4]. Curcumin exhibits anti-inflammatory, anti-oxidant, hypoglycemic, lipid-lowering, anti-proliferation, anti-coagulation, and anti-thrombotic activity via diverse molecular targets [5]. Furthermore, curcumin modulates signaling pathways and regulates the expression of several molecules, including inflammatory cytokines, growth factors, receptors, and transcription factors. Much research has focused on the anti-atherosclerotic role of curcumin; however, the underlying mechanism regulating curcumin-induced protection against atherosclerosis remains unclear [6]. Curcumin can inhibit EMMPRIN, matrix metalloproteinase (MMP)-9, and MMP-13 expression in phorbol myristate acetate (PMA)-induced macrophages, which may exacerbate plaque vulnerability [7]. Numerous papers have demonstrated that curcumin exerts anti-inflammatory activity in lipopolysaccharide (LPS)/interferon (IFN)- γ -treated macrophages via multiple mechanisms. Curcumin strongly inhibits the LPS-induced expression of interleukin (IL)-6 in RAW264.7 cells by blocking the nuclear factor (NF)- κ B and signal transducer and activator of transcription 1 (STAT1) signaling pathways [8]. Furthermore, a curcumin derivative suppressed nitric oxide (NO), tumor necrosis factor- α (TNF- α), and IL-1 β expression by inhibiting the mitogen activated protein kinase (MAPK)/NF- κ B pathways in IFN- γ /LPS-stimulated macrophages [9]. Nevertheless, it remains unclear if curcumin regulates macrophage polarization during atherosclerosis.

Toll-like receptor 4 (TLR4), a pattern recognition receptor, plays a key role in inflammation and the immune system. TLR4 is primarily expressed in human monocytes and recognizes multiple microbial and endogenous molecules via their molecular patterns, including LPS, heat-shock proteins 60 and 70, fibrinogen, and oxidized lipids [10, 11]. Upon binding, recruitment of extracellular and intracellular adaptor molecules into TLR4 complexes triggers kinase signaling cascades, ultimately regulating the expression of inflammatory cytokines via the activation and nuclear translocation of NF- κ B. IFN- γ may synergistically enhance macrophage activity by augmenting TLR-induced activation of inflammatory cytokines, including TNF- α , IL-6, and IL-12 [12, 13]. Recent evidence suggests that TLR4-mediated inflammation is a critical pathogenic link between innate immunity and atherosclerosis. The Asp299Gly TLR4 polymorphism attenuates receptor signaling and diminishes the inflammatory response, thereby decreasing the risk of atherosclerosis [14].

TLR4 is also abundantly expressed in atherosclerotic plaques in humans and animal models, contributing to atherothrombosis initiation and progression [15, 16]. Furthermore, global TLR4 deficiency reduces adipose tissue inflammation by shifting macrophage polarization toward the M2 phenotype [17]. However, little attention has been focused on therapeutic measures targeting TLR4 and its downstream pathways for effective modulation of polarized macrophage phenotype. Thus, we investigated the molecular action of curcumin, a potential anti-atherosclerotic drug, on polarized macrophages, and evaluated whether the TLR4-MAPK/NF- κ B pathways modulated macrophage polarization.

Materials and Methods

Cell culture and treatment

The human monocytic THP-1 cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco-BRL, Gaithersburg, MD, USA) at 37°C in a 5% CO₂ incubator. THP-1 cells were treated with 100 nM PMA (Sigma, St. Louis, MO, USA) for 48 h to induce cellular differentiation into macrophages. After differentiation, non-attached cells were removed, and the adherent macrophages were washed three times. The PMA-differentiated THP-1 cells were incubated for 24 h with LPS (1 μ g/ml) and INF- γ (20 ng/ml) in the presence of varying curcumin concentrations (0, 7.5, 15, and 30 μ M, Sigma), with or without the JNK inhibitor SP600125, the ERK inhibitor SCH772984, or the p38 inhibitor Skepinone-L (Selleck Chemicals, Houston, TX, USA).

³H]thymidine (3H-TdR) incorporation assay

Curcumin-induced growth inhibition in differentiated cells was measured using a ³H-TdR (China Institute of Atomic Energy, Tuoli, China) incorporation assay. THP-1 cells were seeded in a 96-well plates at a density of 1 \times 10⁶ cells/ml. After PMA-induced differentiation, cells were cultured for 24 h in the presence of curcumin. Cells without any treatment were used as a control. Twelve hours before harvesting, 1 μ Ci ³H-TdR was added to each well. Macrophages were harvested onto glass fiber filter paper, and the counts per minute (cpm) were determined using a Wallac TriLux 1450 MicroBeta microplate scintillation counter (PerkinElmer, Waltham, MA, USA). The experiment was repeated three independent times.

TLR4 small interfering RNA (siRNA) transfection

siRNAs targeting the TLR4 gene were designed and synthesized by Invitrogen (Grand Island, NY, USA). The sequence for the siTLR4 was as follows: CAT CGT TTG GTT CTG GGA GAA TTT A. Lipofectamine RNAiMAX (Invitrogen) was utilized to transfect siTLR4 into macrophages according to the manufacturer's protocol. Complexes were incubated for 72 h at room temperature. Macrophages transfected with a non-target control siRNA were used as controls.

RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Total RNA was prepared from macrophages with TRIzol Reagent (Invitrogen), and the concentration was measured using the absorbance at 260 nm. The total RNA was reverse-transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, USA). Real-time PCR was performed using SYBR Premix Ex Taq (TaKaRa) following the manufacturer's instructions. Samples were run in triplicate. The real-time PCR conditions were carried out on the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as follows: 50°C, 2 min; 95°C, 3 min; followed by 40 cycles of 95°C for 12 s and 62°C for 1 min. Semi-log amplification curves were analyzed using the 2^{- $\Delta\Delta$ CT} method, and the relative expression of each gene was normalized to that of β -actin. The PCR primer sequences are listed in Table 1.

Table 1. Primer sequences used for real-time PCR

Gene	Primer	Sequences(5'-3')	Product size/bp
TNF α	Forward	ATTGCCCTGTGAGGAGGAC	104
	Reverse	TGAGCCAGAAGAGGTTGAGG	
IL-6	Forward	CTTCGGTCCAGTTGCCTTCT	120
	Reverse	GTGAGTGGCTGTCTGTGTGG	
IL-12	Forward	CTTGTGGCTACCCTGGCTCT	151
	Reverse	GAGTTTGTCTGGCCTTCTGG	
β -actin	Forward	CTCCATCCTGGCGCTGT	268
	Reverse	GCTGTACCTTCACCGTT	

Enzyme-linked immunosorbent assay (ELISA)

After stimulation and sample treatment, the cell culture supernatants were collected and analyzed for TNF α , IL-6, and IL-12B (p40) secretion using ELISA kits (Abcam, US) following the manufacturer's instructions. All samples were assayed in triplicate. The data were acquired by measuring the absorbance at 450 nm on a microplate reader 3550-UV (Bio-Rad, Hercules, CA, USA). The concentration of TNF α , IL-6, and IL-12B (p40) in each sample was calculated using standard curves.

Protein extraction and immunoblotting

Western blot analysis was performed according to the standard procedures. Briefly, macrophages were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma) at 4°C for 30 min, and then subjected to 10,000 rpm centrifugation at 4°C for 20 min. Protein concentration was determined by BCA protein assay kit. The lysates were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes, and blocked with 5% skim milk. The blots were hybridized with primary antibodies (Cell Signaling Technology, Beverly, MA, USA) at 4°C overnight. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 h (1:5000 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) and quantified by densitometry using GelPro 3.2 software. β -actin expression was used as an internal control.

Flow cytometry

Cultured cells were washed with cold PBS buffer and incubated with FITC-conjugated anti-human leukocyte antigen (HLA)-DR (BD Pharmingen, San Jose, CA, USA) or isotype control antibody at 4°C for 30 min. For intracellular CD68 staining, harvested macrophages were trypsinized and incubated with PerCP/Cy5.5-conjugated anti-human CD68 (BD Pharmingen) at 4°C for 30 min. The cells were then centrifuged at 10000 rpm at 4°C for 5 min and re-suspended in PBS. The expression of HLA-DR and CD68 were determined by flow cytometry to identify the macrophage polarization phenotype. Fluorescence was analyzed, quantifying 1×10^4 events using gates to exclude non-viable cells. Data acquisition and analysis were performed with FACScan system using CellQuest software (BD Bioscience).

Statistical analysis

The statistical program SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Data were found to be normally distributed and were expressed as mean \pm standard deviation. Comparisons between two groups were analyzed by the Student's *t*-test, while comparisons between more than two groups were analyzed by analysis of variance. A *p*-value less than 0.05 was considered statistically significant.

Results

Effect of curcumin on PMA-induced macrophage differentiation

To evaluate curcumin-induced cytotoxicity in PMA-differentiated cells, we evaluated macrophage growth inhibition following curcumin treatment (0, 7.5, 15, 30 μ M) by 3 H-TdR incorporation assay. As shown in Fig. 1A, there was no significant difference in cell growth following curcumin treatment at any dose. Furthermore, curcumin did not significantly affect macrophage polarization (Fig. 1B-C). In contrast, curcumin dose-dependently inhibited TNF α , IL-6, and IL-12B (p40) secretion and gene expression (Fig. 1D-E). Remarkably, treatment with 30 μ M curcumin reduced inflammatory cytokine production by 78.8% ($p < 0.001$), whereas gene expression was inhibited by 44.4%-59.8% ($p < 0.001$) as compared to the control.

Effect of curcumin on LPS/IFN- γ induced M1 macrophages

To explore the correlation between curcumin treatment and macrophage polarization, we treated LPS/IFN- γ -induced M1 macrophages with curcumin (0, 7.5, 15, 30 μ M) and analyzed the corresponding macrophage phenotype. Consistent with previous studies, we used CD68⁺HLA-DR⁺ and CD68⁺HLA-DR⁻ as markers for M1 and M2 macrophages, respectively [18, 19]. Our results indicated that curcumin dose-dependently inhibited the

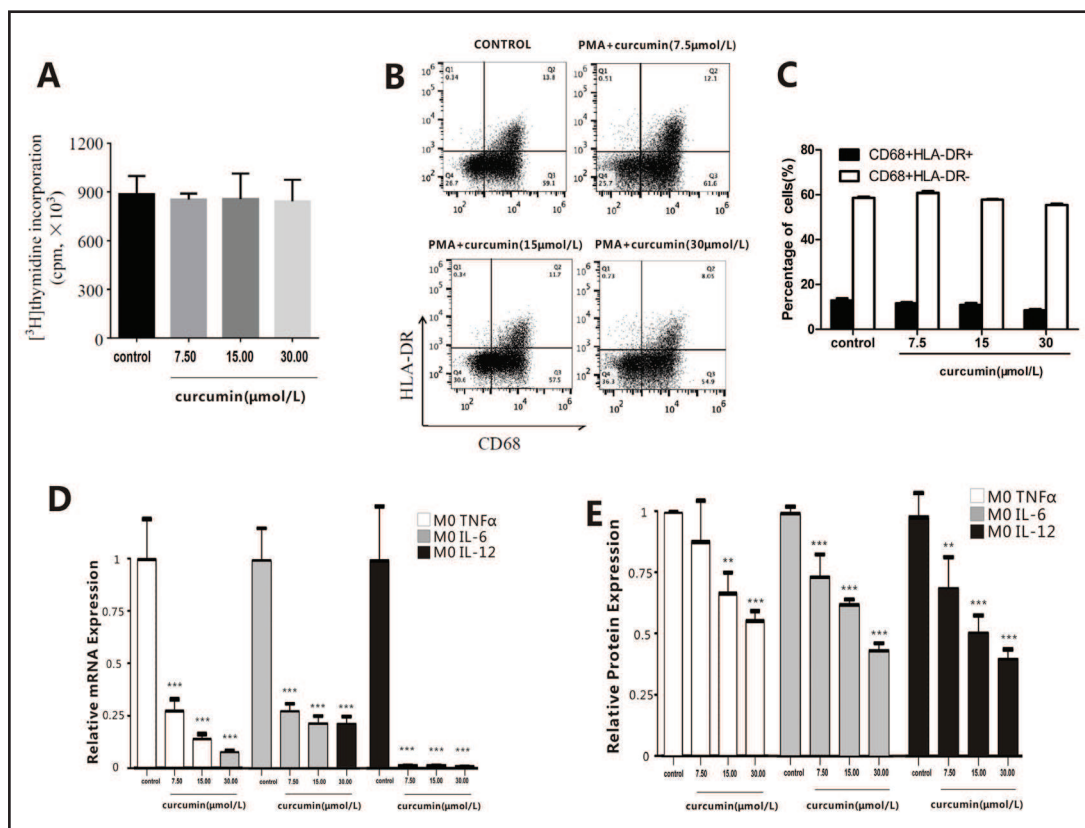


Fig. 1. Effect of curcumin on PMA-differentiated cells. Growth inhibition of macrophages treated with different concentration of curcumin at 0, 7.5, 15, 30 $\mu\text{mol/L}$ for 24 h were detected by ^3H -TdR incorporation assay (A), and the percentage of $\text{CD68}^+\text{HLA-DR}^+$ and $\text{CD68}^+\text{HLA-DR}^-$ cells, which were recognized as M1 and M2 macrophage markers, were analyzed by flow cytometry (B,C). The mRNA level and protein level were measured by Real-time PCR and ELISA, respectively (D, E). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control group.

M1 macrophage polarization ($p < 0.05$). Interestingly, the proportion of $\text{CD68}^+\text{HLA-DR}^+$ M1 macrophages decreased, while the $\text{CD68}^+\text{HLA-DR}^-$ M2 phenotype increased (Fig. 2A-B). Additionally, curcumin treatment dose-dependently inhibited $\text{TNF}\alpha$, IL-6, and IL-12B ($p40$) protein and mRNA levels in LPS/ $\text{IFN-}\gamma$ -induced M1 macrophages (Fig. 1C-D). These data suggest that curcumin may switch the pro-inflammatory M1 cells towards an M2 phenotype.

Effect of curcumin on the MAPK/ $\text{NF-}\kappa\text{B}$ pathway

To gain insight into the potential mechanism of curcumin-mediated macrophage polarization regulation, we evaluated the effects of curcumin on the MAPK/ $\text{NF-}\kappa\text{B}$ signaling pathway. Macrophages were pretreated with curcumin (30 μM) for 1 h, and incubated with LPS/ $\text{IFN-}\gamma$ for 24 h. Total and phosphorylated p38, ERK1/2, JNK1/2, IKK, $\text{I}\kappa\text{B}\alpha$, and p65 were measured by western blot. LPS/ $\text{IFN-}\gamma$ treatment activated both the MAPK (p38, ERK1/2, and JNK1/2) and $\text{NF-}\kappa\text{B}$ ($\text{I}\kappa\text{B}\alpha$ and p65) pathways (Fig. 3A, B). However, curcumin significantly suppressed the activation of the MAPK/ $\text{NF-}\kappa\text{B}$ signaling pathways, although total p38, ERK1/2, JNK1/2, $\text{I}\kappa\text{B}\alpha$, and p65 expression was unaltered by LPS/ $\text{IFN-}\gamma$ or curcumin. Our results suggest that curcumin abolishes MAPK/ $\text{NF-}\kappa\text{B}$ pathway activation.

TLR4/MAPK pathway regulated macrophage polarization towards M1 phenotype

To further elucidate the underlying mechanisms of curcumin activity, we examined whether the TLR4/MAPK pathway regulates macrophage polarization. LPS/ $\text{INF-}\gamma$ stimulated macrophages were incubated in the presence or absence of MAPK inhibitors. We utilized siTLR4, the JNK inhibitor SP600125, the ERK inhibitor SCH772984, and the

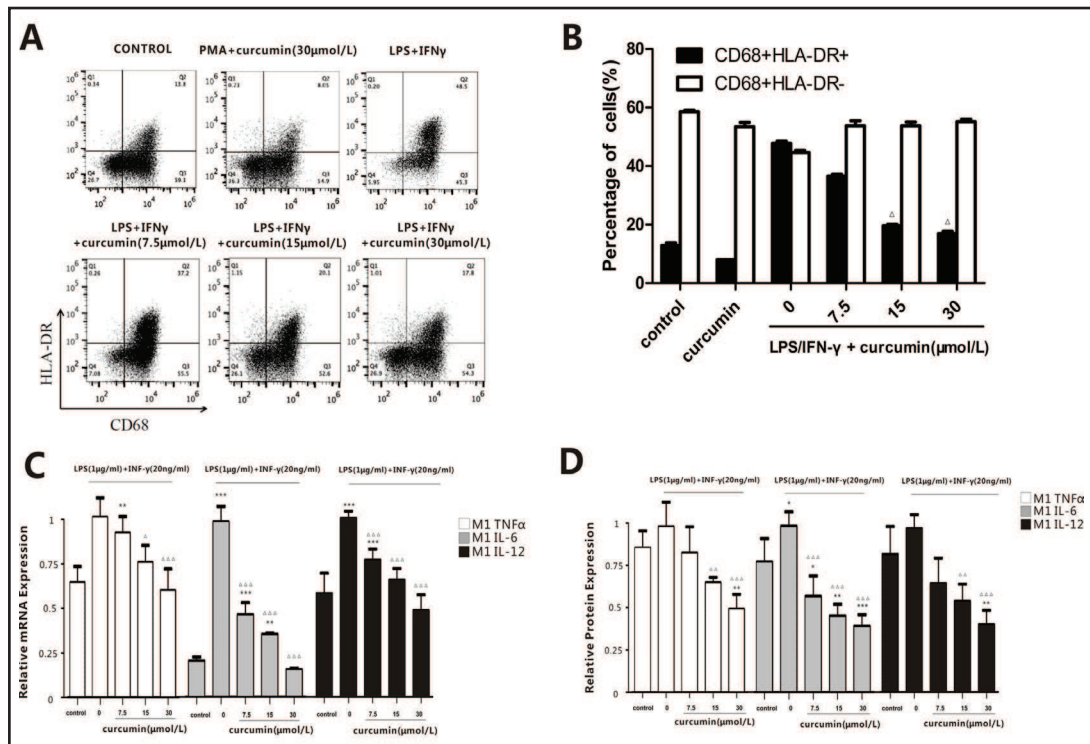
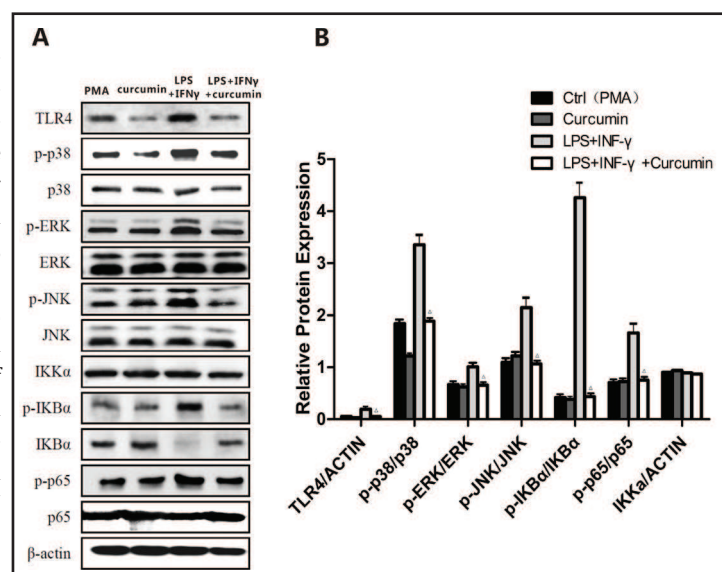


Fig. 2. Effect of curcumin on LPS/IFN- γ induced M1 macrophages. Macrophages were induced by LPS/IFN- γ to be polarized towards M1 phenotype, which were then treated by curcumin at 0, 7.5, 15, 30 $\mu\text{mol/L}$ for 24h and analyzed corresponding phenotype of macrophages by flow cytometry (A, B). Culture medium was collected and the levels of TNF α , IL-6 and IL-12B (p40) were measured with ELISA kits (C). Total RNA was extracted after the same treatment and the levels of TNF α , IL-6 and IL-12B (p40) mRNA were detected by quantitative real-time PCR (D). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control group. $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ vs LPS/IFN- γ induced group.

Fig. 3. Curcumin suppresses MAPK/NF κ B pathway in LPS/IFN- γ -induced macrophages. Macrophages were pretreated with curcumin (30 $\mu\text{mol/L}$) for 1 h in the absence or presence of LPS/IFN- γ for another 24 h. The total and phosphorylation of p38, ERK1/2, JNK1/2, IKK, I κ B α and p65 was measured by Western blot (A, B). Curcumin suppressed expression of TLR4 and phosphorylation of p38, ERK1/2, JNK1/2, I κ B α and p65, but did not impact the expression of IKK in LPS/IFN- γ induced macrophages. $\Delta P < 0.05$ vs LPS/IFN- γ induced group.



p38 inhibitor Skepinone-L to block the TLR4/MAPK pathway at different levels. These effects were evaluated by western blot (Fig. 4A, B). We then conducted flow cytometry in the experimental groups to identify the role of the TLR4/MAPK pathway in macrophage

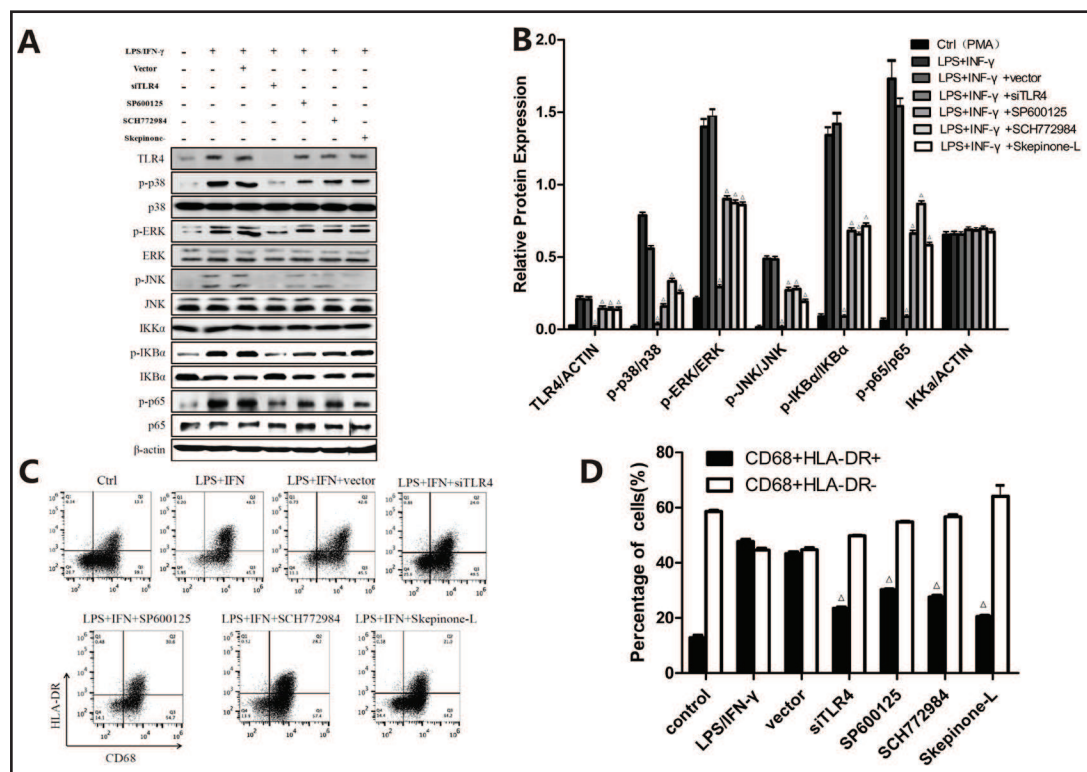


Fig. 4. TLR4/MAPK pathway regulated macrophage polarization towards M1 phenotype. LPS/IFN- γ induced macrophages were cultured in the absence or presence of siTLR4, SP600125 (an inhibitor of JNK), SCH772984 (an inhibitor of ERK) and Skepinone-L (an inhibitor of p38) respectively for 24h. Then, all the cells were harvested for Western blot assay (A). Flow cytometry showed that the proportion of CD68+HLA-DR+ M1 macrophages was respectively dropped by siTLR4, SP600125, SCH772984 and Skepinone-L (C, D). ^aP < 0.05 vs LPS/IFN- γ induced group.

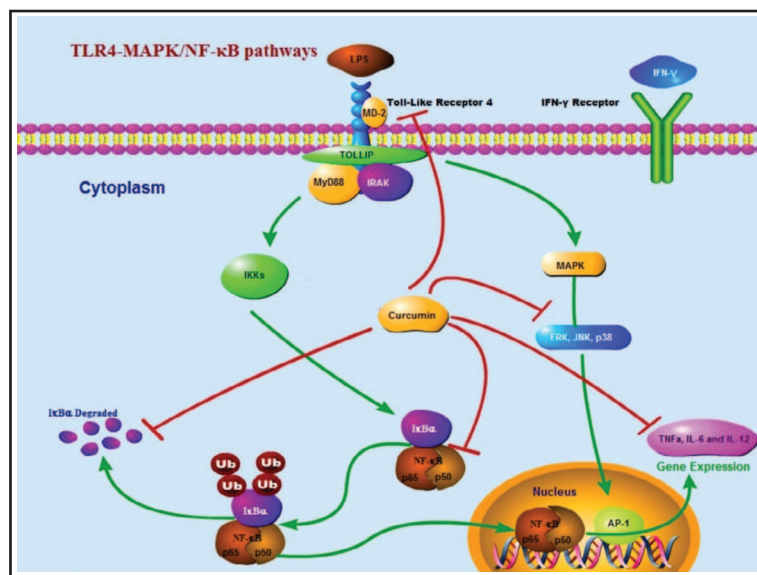
polarization. Our results indicated that the proportion of CD68+HLA-DR+ M1 macrophages was reduced by siTLR4, SP600125, SCH772984, and Skepinone-L (Fig. 4C, D). Taken together, M1 macrophage polarization was dependent of the activation of the TLR4/MAPK signaling pathway, in agreement with our hypothesis that curcumin polarized M1 macrophages towards M2 phenotype via inhibition of the TLR4/MAPK/NF- κ B signaling pathway.

Discussion

Macrophages play an indispensable role in atherosclerosis through a variety of immune functions. Interestingly, macrophages are capable of polarization into specialized phenotypes in response to signals from the local microenvironment. Recently, one study suggests that curcumin could induce the polarization of murine RAW264.7 cells. Curcumin could not only inhibit the M1 inflammation phenotype, but also up-regulate the expression of the M2 phenotype markers [20]. Consistent with previous work, our findings also indicate that curcumin could dose-dependently switch M1 macrophages to an M2 phenotype through TLR4-MAPK/NF- κ B pathway inhibition (Fig. 5).

Polarized macrophages can be broadly classified into two functionally distinct groups. The first group is classically-activated macrophages (pro-inflammatory, M1), whose classical stimuli are Th1-related cytokines, such as IFN- γ , and microbial products, such as LPS. Alternatively-activated macrophages (anti-inflammatory, M2), are polarized by Th2 cytokines, such as IL-4 and IL-13 [21]. M2 macrophages can be further subdivided into three groups:

Fig. 5. A model representing curcumin molecular targets in TLR4-MAPK/NF- κ B pathway. TLR4 stimulation by LPS triggers intracellular signaling cascades, including NF- κ B and MAPK pathways, which ultimately leads to the expression of inflammatory cytokines such as TNF- α , IL-6, and IL-12. Curcumin exerts its anti-inflammatory effect by inhibiting TLR4 expression, the phosphorylation of ERK, JNK, p38, and nuclear factor (NF)- κ B in macrophages. The action of curcumin is indicated by red lines.



M2a (alternatively activated macrophages, AAM), M2b, and M2c (regulatory macrophages) [22]. M1 and M2 macrophage populations can be differentiated based on the expression of cell-surface markers. Among these, CD11c, CD86, MHC-II, and the macrophage receptor with collagenous structure (MARCO) are characteristic of the M1 phenotype. Alternatively, the scavenger receptor, mannose receptor (CD206), arginase I, dectin-1 (CLEC7A), and CD163 are upregulated in M2 macrophages. This may seem inconsistent with our selection of markers to identify M1 and M2 macrophages. Indeed, many mouse-specific markers are rarely expressed in human polarized macrophages. The discordant mechanism involved might be due to different experimental conditions, cell types, or regulation variability between species. Thus, we identified macrophage populations using two cell-surface markers, CD68 and HLA-DR, as previously described.

The remarkable plasticity and distinct physiology of macrophages can give rise to different homeostatic activities. Classically activated macrophages produce a repertoire of pro-inflammatory mediators, such as reactive oxygen species and cytokines (TNF α , IL-6, IL-12). They are engaged in inflammatory, microbicidal, and tumoricidal activities; however, M1 activation must be tightly controlled in order to avoid host-tissue damage. In contrast, alternatively activated macrophages can promote Th2 immune responses and contribute to the resolution of inflammation, increased phagocytic activity, and tissue repair [23]. Therefore, differential modulation of macrophage phenotype profoundly affects the progress of atherosclerosis. In particular, recent evidence suggests that curcumin could prevent RAW264.7 cells from polarizing to M1 macrophages through the activation of I κ B α , while it promotes the transition from M0 or M1 phenotype to M2 macrophages [20]. Consistent with previous work, our findings also indicate that curcumin could dose-dependently switch M1 macrophages to an M2 phenotype. It remains unclear whether individual macrophage populations are manipulated or selectively targeted by therapeutics. The major issue preventing therapeutic manipulation of macrophage phenotypes in atherosclerotic lesions is that very little is known about the intracellular signaling pathways and transcription factors involved in macrophage activation [24]. It should be noted, however, that our findings are based on the measurement of TLR4-dependent activation of signal transduction pathways.

Mitogen-activated protein kinases (MAPKs) are a group of protein kinases downstream of TLR4 that regulate diverse functions, including inflammatory responses, differentiation, and apoptosis. The MAPK signaling pathway includes a three-tiered kinase core, whereby a MAPKKK activates a MAPKK, which in turn activates a MAPK, such as ERK, JNK, and p38 MAPK [25]. These kinases mediate phosphorylation and ubiquitination of various substrates,

eventually resulting in expression of several immunomodulatory cytokines. NF- κ B is a transcription factor that consists of five genes: NF- κ B 1 (p50/p105), NF- κ B 2 (p52/p100), RelA (p65), c-Rel, and RelB. NF- κ B consists of a heterotrimer of p50, p65, and an inhibitory protein, I κ B α , in the cytoplasm at resting state. IKK-mediated phosphorylation of I κ B and subsequent degradation by the proteasome allows the p50-p65 heterodimer to translocate into the nucleus, inducing the transcription of pro-inflammatory cytokines, including TNF- α , IL-6, and IL-12 [26, 27]. As a potent agonist of TLR4, LPS facilitates not only the expression of TLR4, but also the phosphorylation of ERK, p38, and JNK, and the activation of NF- κ B [28]. In this study, we investigated the involvement of TLR4 mediated pathways in macrophage polarization. Although further evidence in support of these observations is required, it is tempting to speculate that activation of TLR4 and its downstream pathways triggers the phenotypic switch of macrophages from a quiescent population to an inflammatory population.

Although there is controversy regarding how curcumin affects the TLR4-mediated signal transduction pathway, there seem to be general agreement that curcumin attenuate LPS-induced inflammation by inhibiting the overexpression of TLR4, the dimerization of the TLR4 receptor, the phosphorylation of MAPK signal transduction, the degradation of I κ B α , and the nuclear translocation of NF- κ B (p65) [29, 30]. It is also consistent with our observations in this study. In addition, it has been well documented that curcumin serves as a TLR4 antagonist, binding to myeloid differentiation protein 2 (MD-2) to compete with LPS *in vitro* [31]. In a more detailed analysis of the molecular mechanism of action, curcumin was shown to have α , β -unsaturated carbonyl structural motifs conferred by the Michael addition, which interacts with a catalytically active cysteine residue of thioredoxin reductase in TLR4. Thus, curcumin may inhibit enzyme activity via inhibition of both ligand-induced and ligand-independent dimerization of TLR4 [32].

Additionally, curcumin is hypothesized to selectively block JNK and NF- κ B activation by various stimuli, further implying that curcumin may interfere with regulators at the same point upstream of MAPKKK [33]. These results contrast our findings, which suggest that curcumin completely suppressed LPS/IFN- γ activated the TLR4/MAPK/NF- κ B pathways (Fig. 4). The discrepancies between their observations and ours are likely due to differences in cell type and stimulation conditions. Similarly, it was previously reported that curcumin could suppress monocyte chemoattractant protein-1 (MCP-1) production induced by oxidized low-density lipoprotein (ox-LDL) in macrophages via the JNK and NF- κ B pathways [30]. Curcumin also suppressed LPS-induced inflammatory responses in rat vascular smooth muscle cells through the inhibition of the TLR4-MAPK/NF- κ B pathways. Although the mechanisms underlying macrophage heterogeneity remain elusive, it is reasonable to presume that TLR4, together with its downstream signaling pathways, is a candidate switch between M1 and M2 phenotypes. The potential role of TLR4 for therapeutic intervention in atherosclerosis is just beginning to be appreciated [34, 35]. The anti-atherosclerosis effect of curcumin via modulation of the TLR4-mediated signaling pathways associated with M1 activation has not been investigated. Further studies are warranted to better understand how curcumin modulates macrophage polarization and function, which is also critical for targeted atherosclerosis therapy. It is of note that previous work has focused on the anti-inflammatory effect of curcumin in macrophages. In contrast, our present findings suggest that curcumin-mediated macrophage polarization is worthy of attention.

It should be noted that there are some limitations in the present study. First, we employed macrophages derived from THP-1 cells, rather than other cell types. Future studies should investigate curcumin-mediated macrophage polarization in other macrophages. Second, we focused primarily on the TLR4-MAPK/NF- κ B pathway, despite other potential mechanisms related to macrophage polarization. Additional experiments with TLR4-independent stimulations should also be performed to further verify the direct effect of curcumin on MAPK/NF- κ B pathway. Third, we utilized only CD68 and HLA-DR as surrogate markers to discriminate macrophages. More definitive biochemical markers to differentiate macrophage populations are still needed. Finally, *in vivo* experiments using knockout mouse

models are necessary to further validate the effect of curcumin-mediated macrophage polarization.

Taken together, plasticity is a hallmark of the mononuclear phagocyte system, which can be a surrogate biomarker of atherosclerosis and therapeutic efficacy following therapy. Further work is required to provide deeper insight into the major molecular mechanisms responsible for modulation of macrophage phenotype by curcumin. In this context, a potential phenotypic switch in macrophage polarization by curcumin may pave the way for future clinical research and application of this phytochemical constituent for prevention and treatment of atherosclerosis.

Disclosure Statement

The authors declare that they have no potential conflict of interest.

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