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Curcumin inhibits 19-kDa lipoprotein of *Mycobacterium tuberculosis* induced macrophage apoptosis via regulation of the JNK pathway

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ABSTRACT

Recently, synthetic curcumin analogs are reported as potential active compounds against *Mycobacterium tuberculosis* (MTB). During the process of MTB infection, macrophages show increased apoptosis. The candidate virulence factors such as 19-kDa lipoprotein secreted by the MTB (P19) strongly influences macrophages by activation of Toll-like receptor 2 (TLR2) and mitogen-activated protein kinases (MAPKs). It has been reported that curcumin could affect the apoptosis of tumor cells via regulation of MAPKs. However, its effect on the P19-induced apoptosis of macrophages is unclear. This study investigates the effect of curcumin on the MAPKs signaling and apoptosis in human macrophages. The results showed that curcumin and P19 induced macrophage apoptosis in a time- and dose-dependent manner Low doses of curcumin (10 and 20 μM) protected macrophages from P19 induced apoptosis, accompanied by decreased production of cytokines and reduced activation of the c-Jun amino-terminal kinase (JNK) and p38 MAPK. The protective effect of curcumin on P19 induced apoptosis of macrophages were enhanced by treatment with the JNK-specific inhibitors, whereas SB203580, the inhibitor of p38 MAPK had no effect. Curcumin had no effect on the activity of extracellular signal-regulated protein kinase (ERK). Taken together, our data show that the JNK pathway, but not the p38 or ERK pathway, plays an important role in the protective effect of curcumin against P19 induced macrophage apoptosis, and regulation of the JNK pathway may partially elucidate the anti-tuberculosis activity of curcumin.

1. Introduction

Tuberculosis, caused by *Mycobacterium tuberculosis* (MTB), is amongst the foremost infectious diseases. One-third of the world's population is affected by MTB and one-tenth of the infected population will eventually develop active tuberculosis [1]. Macrophages could eliminate infectious agents (such as bacteria), particles and specific molecular complexes. However, in fact, macrophages are also the principal target for mycobacteria such as MTB. The macrophages infected with MTB will show an increase in apoptosis, which is important for the pathogenesis of tuberculosis. Thus, identification of specific factors from MTB responsible for inducing apoptosis will provide insight into the pathogenesis of tuberculosis, and may shed some light on providing novel strategies for the prevention or treatment of tuberculosis.

There are approximately 100 open reading frames identified in the MTB genome that possess a characteristic amino-terminal acylation motif [2]. Synthetic bacterial lipoproteins could induce the apoptosis of macrophages through the Toll-like receptors (TLRs) dependent pathway, and the 19-kDa lipoprotein of *M. tuberculosis* (P19) may be an MTB apoptosis-inducing factor [3]. P19 is an abundantly expressed cell wall-associated immunodominant, and it could stimulate the proliferation of $CD4^+$ T cells and trigger the upregulation of multiple cytokines such as IL-2, IFN-γ, and IL-12 and intracellular signaling events such as the MAPK cascades [4,5]. Recently, Sanchez et al., have revealed that P19 could induce macrophage apoptosis though both extrinsic (TLR2 dependent) and intrinsic (the mitochondrial pathway) mechanisms [6].

Curcumin, a plant-derived natural polyphenol, is recently reported as a promising anti-tuberculosis drug. The synthetic curcumin analogs are also active compounds against MTB, whereas the anti-tuberculosis mechanism of curcumin remains largely unknown [7,8]. Previous studies have shown that curcumin could inhibit inflammation, oxidation and induce cell apoptosis [9–12]. Notably, curcumin can also inhibit the gene expression and

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function of TLR2 possibly via an oxidative process [13], suggesting it may play a role in macrophage apoptosis induced by MTB.

In the present study, we show that both P19 and curcumin induce macrophage apoptosis in a time- and dose-dependent manner. However, curcumin could inhibit P19-induced macrophage apoptosis at low doses though a JNK-dependent pathway. Our study may shed some light on elucidating the anti-tuberculosis mechanism of curcumin.

2. Materials and methods

2.1. Reagents and antibodies

Curcumin was obtained from Sigma–Aldrich (Sigma, St. Louis, MO), dissolved in DMSO, and stored at −20 °C. The final concentration of DMSO in all experiments was less than 0.1%. The JNK inhibitor SP600125 and p38 inhibitor SB203580 were purchased from Cayman Chemicals (Ann Arbor, MI). Cell culture reagents, medium and antibiotics were all obtained from GIBCO BRL (Rockville, MD). The antibody against phospho-ERK (Thr202/Tyr204), total ERK, phospho-JNK (Thr183/Tyr185), total JNK, phospho-p38 (Thr180/Tyr182), total p38, TLR2 and GAPDH were purchased from Cell Signaling Technology (Danvers, MA).

2.2. Bacterial strains and P19 isolation

Lyophilized *M. tuberculosis* H37Rv (ATCC 25618) was obtained from American Tissue Culture Collection (ATCC, Manassas, VA), reconstituted and used as described previously [14]. Purified MTB 19-kDa lipoprotein (P19) was obtained as described previously [15]. In brief, cell-wall fractions were obtained by sonication of resuspended MTB H37Rv in iced water (20 kHz, five cycles, 5 min each). Forty micrograms of protein was dissolved in a reducing sample buffer (0.05 mM EDTA, 0.1% SDS, 1% glycerol, 10% 2-mercaptoethanol, and 0.5 mM/mL Tris–HCl, pH 6.8), heated for 5 min at 95 °C and loaded into a 12% SDS–PAGE gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane and stained with ponceau red to identify the 19 kDa band, and the identity of this band was confirmed in parallel blots with the IT-19 mAb. Then the band was excised, solubilized in DMSO and precipitated with a carbonate/bicarbonate sodium buffer (0.05 M, pH 9.6). The pellet was rinsed thrice with phosphate buffered solution (PBS, pH 7.4) and stored at −20 °C. The concentration of the protein was determined with the Bradford method (Bio-Rad, Hercules, US).

2.3. Cell cultures and P19 infection

Peripherial blood mononuclear cells (PBMCs) were isolated from buffy coat blood sample deriving from a healthy donor using Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation. Experiments were performed according to the institutional regulation of the ethics committee of the Xinxiang Medical University (Xinxiang, China). $CD14^{+}CD16^{+}$ monocytes were enriched by negative selection using the EasySep™ Human Monocyte Enrichment Kit without CD16 Depletion (StemCell Technologies) according to the manufacturer's instructions. Purified monocytes were adjusted to a cell density of 1×10^6 cells/ml and cultured at 37 °C in 5% CO_2 for 6 days. Then, the PBMC-derived monocytes were differentiated to generate mature macrophages, using media consisting of RPMI completed with 1% non-essential amino acids, 1% sodium pyruvate, 0.1% 2-mercaptoethanol, 1% penicillin/streptomycin, 10% fetal calf serum and 50 ng/ml macrophage colony-stimulating factor (M-CSF).

For P19 infection, macrophages were placed in 12-well plates with glass coverslips at a density of 5×10^5 cells/ml for 24 h. The macrophages were then washed and incubated for an additional 18 h in the medium with 0.1% FBS. The macrophages were washed with prewarmed RPMI-1640 medium and replaced with 1 ml of RPMI-1640 supplemented with 10 mM HEPES and 0.4% human serum albumin, and then a series of concentrations of P19 were added to the macrophages. Assays were performed after different time periods.

2.4. The cell viability assays

The cytotoxic effect of P19 and curcumin on macrophages was evaluated using the MTT assay. Briefly, macrophages (1×10^4 cells/well) were seeded into a 96-well culture plate. After adherence, cells were treated with various concentrations of P19 or curcumin, JNK inhibitor SP600125 (30 μM) or p38 inhibitor SB203580P38 (20 μM) and incubated at 37 °C for various time periods. Then the cells were incubated with 100 μl (0.5 mg/ml) of 3-(4,5-Dimethyl-2-Thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Aldrich-Fluka, St. Louis, MO) for 4 h at 37 °C. The formazan crystals were solubilized with DMSO, and the absorbance was measured at 490 nm with a microplate reader (Tecan, Switzerland).

2.5. Cell apoptosis assays

The apoptosis of macrophages in the presence of P19 or curcumin was determined using the Annexin V/propidium iodide (PI) apoptosis kit (Sigma–Aldrich, St. Louis, MO) according to the manufacturer's instructions. Briefly, the macrophages were exposed to P19 (5, 10 or 20 μg/ml) or curcumin (10, 20, 40 and 80 μM) for 48 h. After washing, 1×10^5 cells suspended in 100 μl of Annexin binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) were mixed with 1 μl of FITC-conjugated Annexin V antibody. The mixture was incubated for 15 min at room temperature in dark and analyzed by flow cytometry. PI (2.5 μl) was added to the cell suspension just before analysis. The relative number of the cells that were Annexin V-positive and/or PI positive was determined.

2.6. Cytokine assays

The concentrations of IL-6 and TNF-α in the supernatant of the cell culture were determined by using the human enzyme linked immunosorbent assay (ELISA) (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

2.7. Recombinant adenoviral vectors

The recombinant adenoviral vectors expressing a dominant-negative mutant (Thr183A/Tyr185F) of JNK1 (Ad-DN JNK1) was purchased from Cell Biolabs (catalog No. ADV-115) and was rendered kinase-dead by these two mutations (Ala183/Phe185) within the JNK kinase domain. Adenovirus expressing β-galactosidase (Ad-LacZ) was used as the control and to evaluate the infection efficacy and gene expression. Macrophages were infected with the adenovirus (at a concentration of 10^{10} infectious units/ml) for 48 h to allow expression.

2.8. Western blot

For Western blot, P19 and curcumin were incubated with the macrophages for 48 h. The macrophages were rinsed with prewarmed PBS and lysed in an ice-cold RIPA buffer containing protease inhibitors. The homogenate was incubated on ice for 20 min

and centrifuged at 13,000*g* for 20 min at 4 °C. Then, the supernatant was collected and quantified using the Bradford method (BioRad, Hercules). The whole cell lysates were subjected to 12% SDS–PAGE, subsequently blotted onto a PVDF membrane. The membrane was incubated with the specific antibodies. GAPDH was used as an internal control. The quantitation of protein bands was performed by densitometry using the QuantityOne software (BioRad, Hercules).

2.9. Statistics

All data were analyzed using the commercially available software package SPSS 13.0 (SPSS Inc., USA). All data are expressed as mean ± SEM from three independent experiments. One- or two-way ANOVA with Bonferroni corrections was used to compare the means of three or more groups in the analysis of cell viability, apoptosis, cytokine production and protein expression. The phosphorylation levels of ERK, JNK and p38 were expressed as phosphorylated protein/total protein. P value of <0.05 was considered to be statistically significant. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3. Results

3.1. P19 and curcumin decrease macrophage viability and induces macrophage apoptosis

P19 inhibited the viability of macrophages in a concentration- and time-dependent manner [$F_{\text{concentration}(3,\ 43)} = 53.9$, $P < 0.0001$; $F_{\text{time}(4,\ 43)} = 22.8$, $P < 0.0001$; $F_{\text{interaction}(12,\ 43)} = 6.09$, $P < 0.0001$] (Fig. 1A). After 48 h of incubation, P19 significantly inhibited the cell viability (inhibition rate of 10 μg/ml: 28.2%; 20 μg/ml: 65.5%). Similarly, curcumin was also found to inhibit the cell viability in a concentration- and time-dependent manner [$F_{\text{concentration}(4,\ 74)} = 73.5$, $P < 0.0001$; $F_{\text{time}(4,\ 74)} = 10.9$, $P < 0.0001$; $F_{\text{interaction}(16,\ 74)} = 2.74$, $P < 0.0001$] (Fig. 1B). Although high concentrations of curcumin (40 and 80 μM) showed a higher inhibition rate of cell viability, low concentrations of curcumin (10 μM and 20 μM) did not show significant inhibition rate. The apoptosis-inducing activity of P19 and curcumin was also evaluated. The representative flow cytometry analysis of apoptosis is shown in Fig. 1C. High concentrations of P19 (20, 10 and 5 μg/ml) and curcumin (80 μM) were found to induce apoptosis in macrophages (Fig. 1D and E). Thus, 20 μg/ml P19, and 10 and 20 μM curcumin were chosen in subsequent experiments.

3.2. Low concentrations of curcumin exerts protective effect against P19-induced viability inhibition and apoptosis in macrophages

Macrophages were exposed to vehicle-control, 20 μg/ml P19 alone or combined with 10 or 20 μM curcumin for 48 h. As expected, P19 significantly induced cell viability inhibition (58.7%; Fig. 1F) and apoptosis (56.1%; Fig. 1G). Strikingly, the P19-induced cell viability inhibition and apoptosis was greatly attenuated in the presence of curcumin (10 μM curcumin: cell viability was 32.2%, and apoptosis was 36.6%; 20 μM curcumin: cell viability was 20.6%, and apoptosis was 16.2%).

3.3. Curcumin attenuated P19-induced cytokine production in macrophages

ELISA was used to measure the levels of IL-6 and TNF-α in macrophages treated with 20 μg/ml P19 in the absence or presence of 10 or 20 μM curcumin for 48 h (Fig. 2A and B). Twenty micrometer of curcumin alone had no effect on the levels of IL-6 and TNF-α. As expected, the levels of IL-6 and TNF-α were significantly increased by P19, whereas the combination of 10 μM curcumin with P19 significantly decreased their levels. Notably, 20 μM curcumin completely offset the P19-induced cytokine production.

3.4. Curcumin attenuates P19-induced phosphorylation of JNK and p38

Next, we measured the expression of TLR2 and the phosphorylation of ERK, JNK and p38 to explore the mechanism underlying the protective effects of the curcumin against the P19-induced inflammatory responses. The representative immunoblots were showed in Fig. 2C. The expression of TLR2 was not significantly altered after treatment with 20 μM curcumin (Fig. 2D), whereas 20 μg/ml P19 significantly increased the TLR2 level. Furthermore, P19-induced TLR2 expression was not affected by co-treatment of 10 μM or 20 μM curcumin.

As shown in Fig. 2E and G, P19 decreased the phospho-ERK level, and increased the phospho-JNK and the phospho-p38 levels. Although 20 μM curcumin alone did not significantly change the p38 or JNK basal levels, the phosphorylation of JNK was significantly decreased by 20 μM curcumin. Moreover, curcumin decreased the P19-induced phosphorylation of JNK and p38 in a dose-dependent manner.

3.5. The blockade of JNK activity, enhanced the protective effect of curcumin against P19-induced inflammatory responses

To investigate the role of JNK and p38 phosphorylation in P19-induced inflammatory responses, we pretreated macrophages with the specific inhibitor for JNK (20 μM SP600125) and p38 (30 μM SB203580) for 1 h prior to the treatment of curcumin and P19. Cytokine production was determined by the ELISA assay. As showed in Fig. 3A and B, the JNK inhibitor significantly decreased the levels of IL-6 and TNF-α, compared with the combination of curcumin and P19. The levels of IL-6 and TNF-α were slightly reduced after treatment with the p38 inhibitor but the difference was not significant.

To independently confirm that these results were not due to nonspecific effects of these pharmacologic inhibitors, we further confirm the above finding by genetic manipulation. Fig. 3C showed that overexpression of Ad-DN JNK1 protein is achieved following infection with the recombinant adenovirus. P19 (20 μg/ml)-induced upregulation of JNK phosphorylation was completely blocked by the overexpression of the Ad-DN JNK1. Macrophages expressing DN-JNK1 showed a significant reduction in both IL-6 and TNF-α level upon P19 stimulation with/without curcumin (Fig. 3D and E). Taken together, these results demonstrated that activation of JNK, but not p38, may play a role in the protective effect of curcumin against P19-induced inflammatory responses.

4. Discussion

Many synthetic bacterial lipoproteins such as P19 are involved in macrophage apoptosis in tuberculosis. In this study, we found that JNK was activated in apoptosis induced by both P19 and curcumin. Macrophage apoptosis induced by P19 and curcumin was time- and dose-dependent. Interestingly, low doses of curcumin could effectively suppress the P19-induced macrophage apoptosis. The effect of curcumin may be JNK-dependent because JNK inhibitors enhanced the protective effect of curcumin against P19-induced inflammatory responses, whereas overexpression of DN-JNK1 reverses the effect of P19 on the growth inhibition of macrophages.

P19 was chosen in this study due to several reasons. First, it is one of the few *Mycobacterial* proteins, which possess acyl groups and mannose residues [16]. Second, P19 behaves as an adhesin

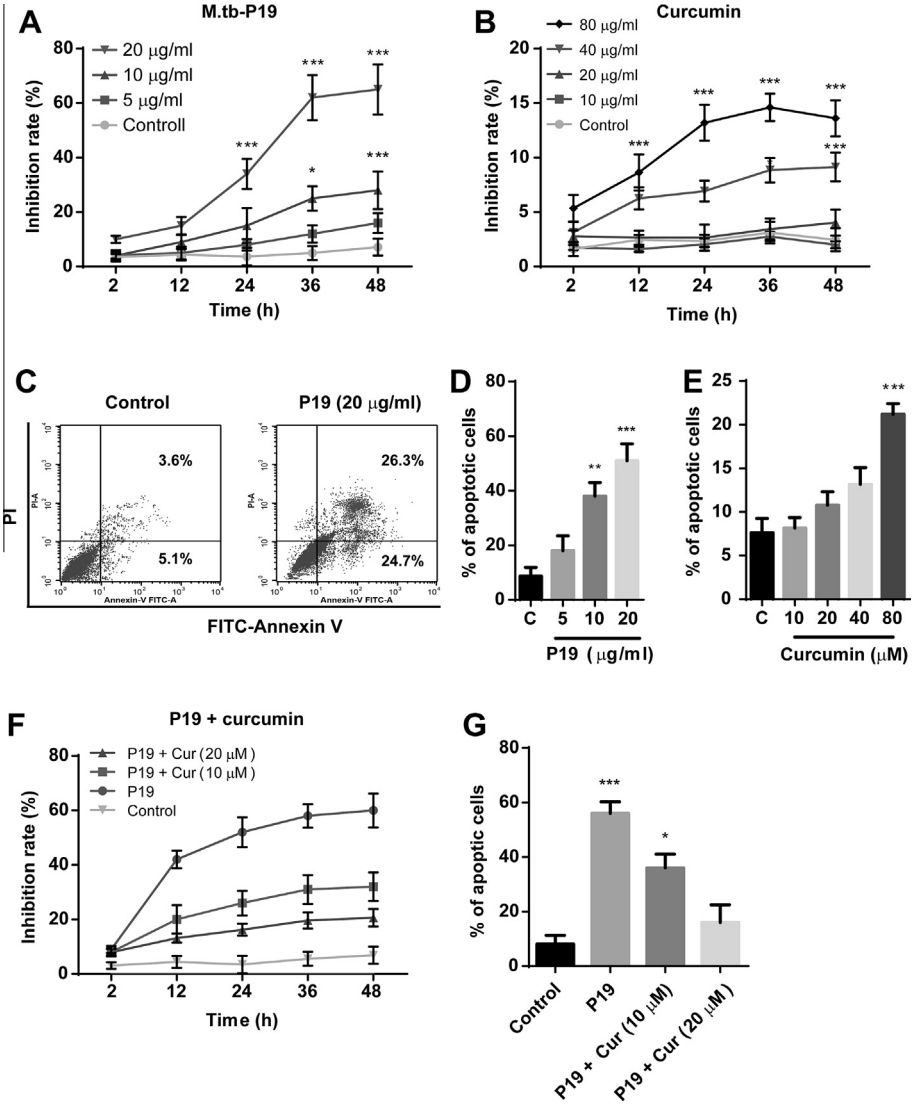

Fig. 1. Effects of P19 and curcumin on the cell viability and apoptosis of macrophages. Cells were treated with (A) 0–20 μg/ml P19 or (B) 0–80 μM curcumin for 48 h and the viability was measured by a MTT assay (C) representative FACS histograms showing Annexin V/PI double staininging macrophages. The apoptosis induced by (D) P19 or (E) curcumin was shown. Then, the macrophages were exposed to 20 μg/ml P19 alone or combined with 10 or 20 μM curcumin for 48 h, (F) the cell viability and (G) apoptosis were determined. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$, compared with the control group.

and interacts with the mannose receptor to promote phagocytosis of mycobacteria [17]. Third, P19 induces T cell-mediated immunity, although it may behave as a TLR2 agonist that downregulates antigen presentation to T cells [18]. Recently, the mechanism underlying P19 induced macrophage apoptosis during MTB infection receives extensive attentions. Generally, P19 promotes macrophage apoptosis through multiple pathways. P19 triggers TLR2 activation and upregulation of death receptors and ligands, followed by a death receptor signaling cascade such as activation of caspase 8 and caspase 3. Pretreatment with neutralizing anti-TLR-2 antibody could inhibit the P19 induced apoptosis in THP-1 cells or TLR-2-expressing Chinese hamster ovary (CHO) cells [19]. Consistently, our results also showed that P19 promoted the expression of TLR2.

Our results suggested that P19, under conditions inducing macrophage apoptosis, caused a strong and persistent activation of JNK and p38 with concomitant inhibition of ERK. In macrophages, a number of microbial pathogens activate three family members of MAPKs: JNK, p38, and ERK, which may play a role in the phagocytosis [20] and intramacrophage survival of microbial pathogens [21,22]. The three family members of MAPKs are also activated by cytokines, growth factors, cellular stress and in the apoptosis induced by several stimuli [23,24]. In particular, JNK and p38, are involved in the caspase-independent apoptosis [25]. Although it is commonly thought that ERK facilitates cell survival while JNK and p38 triggers cell death, the final cell fate is mainly determined by the duration and extent of activation, and balance between activation of different MAPKs family members.

Intriguingly, curcumin is non-cytotoxic to normal cells, and its pharmacological safety is well demonstrated by the fact that people in certain countries have consumed curcumin as a dietary spice for centuries in amounts in excess of 100 mg/day without any side effects [26]. The present study have provided the evidence that curcumin induce macrophage apoptosis in a dose- and time-dependent manner. Although the fact that curcumin could induce cell apoptosis is well established, the exact mechanism accounting for macrophage apoptosis is not yet fully defined. The possible mechanism of curcumin-induced apoptosis involves inhibition of cellular protein kinases including PKC, JNK, and the EGF receptor kinase. Curcumin is also known to activate several apoptosis

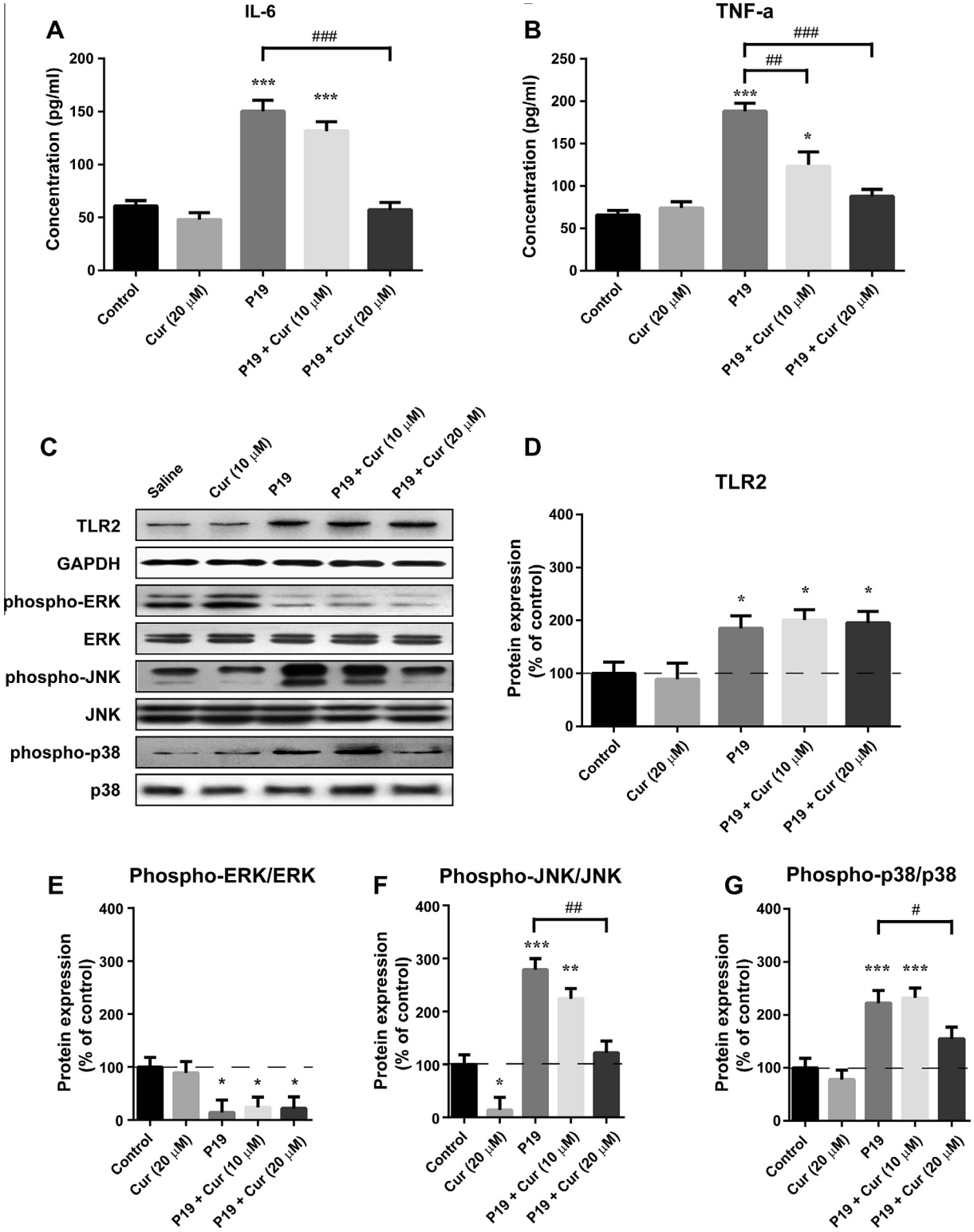

Fig. 2. P19-induced cytokine production and MAPKs activation. After treatment of 20 μg/ml P19 alone or combined with 10 or 20 μM curcumin for 48 h, the levels of (A) IL-6 and (B) TNF-α were measured by ELISA (C) representative immunoblots. The expression of TLR2 (D) and the phosphorylation levels of (E) ERK, (F) JNK and (G) P38 were determined by Western blot. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$, compared with the control group. $^{\#}P < 0.01$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.0001$, compared with the indicated group. Cur, curcumin.

pathways, such as tumor-suppressor gene p53-mediated apoptosis in human carcinoma cell under stress conditions [27]. Mitochondrial dysfunction triggered by enhanced Bax also contributes to curcumin-induced apoptosis [28,29]. Additionally, curcumin can block the NF-κB cell survival pathway [30]. However, the mechanism underlying curcumin-induced apoptosis is mainly restricted to tumor cell line, whereas the mechanism underlying curcumin-induced apoptosis in macrophages still remain unclear. In the

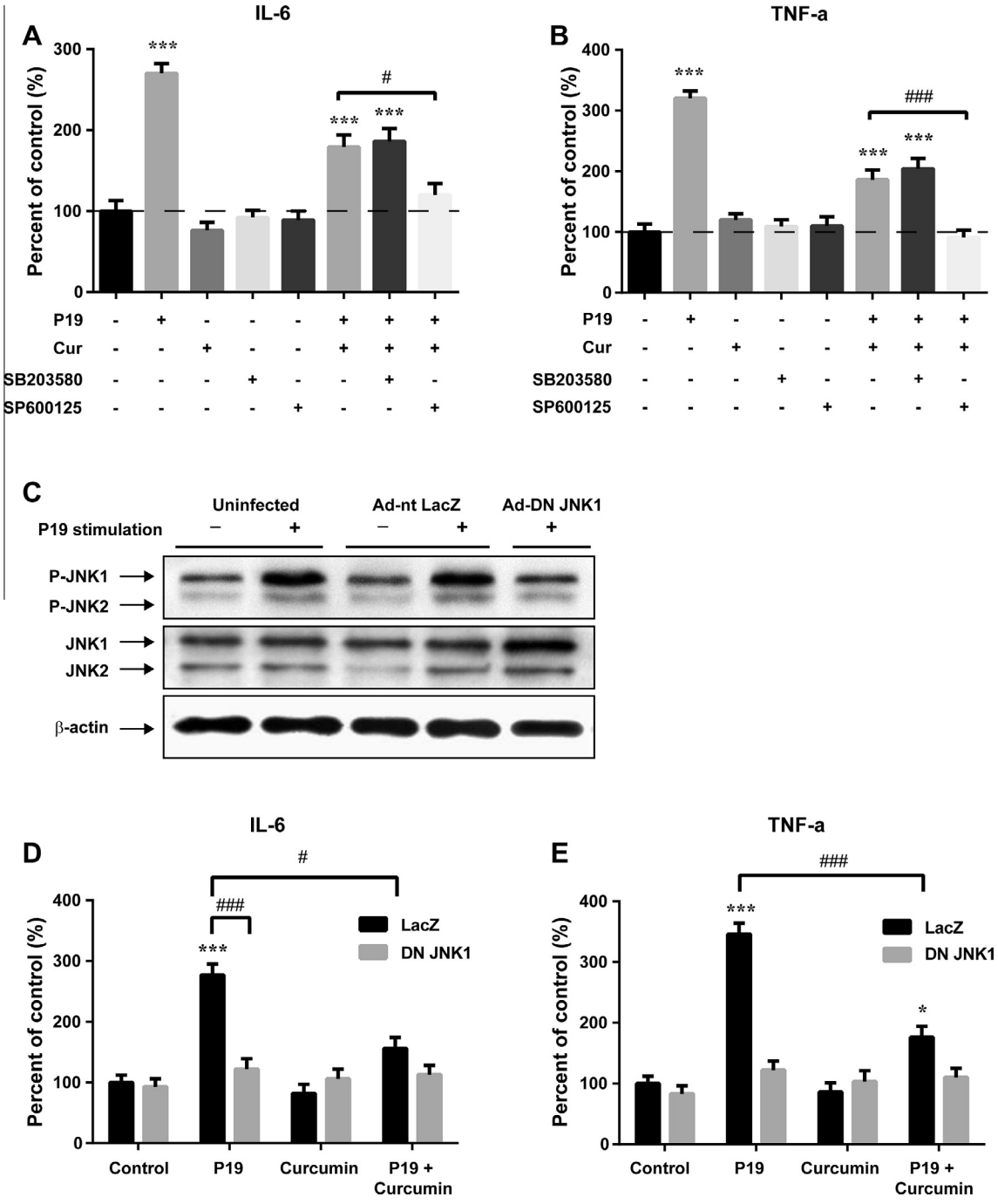

Fig. 3. Blockade of JNK, but not p38, enhanced the protective effect of curcumin against P19-induced inflammatory responses. Macrophages were pretreated for 1 h with and without the JNK inhibitor SB203580 (20 μM) or the p38 inhibitor SP600125 (30 μM) before treating with 20 μg/ml P19 and/or 20 μM curcumin for 48 h. The levels of (A) IL-6 and (B) TNF-α were measured by ELISA. Overexpression of DN JNK1 was achieved by transfection of Ad-DN JNK1. (C) P19 (20 μg/ml)-induced upregulation of phosphor-JNK was completely blocked in macrophages expressing DN JNK1. Macrophages expressing DN JNK1 showed significant reduction in both (D) IL-6 and (E) The level of TNF-α upon P19 stimulation with/without curcumin. $^{*}P < 0.05$, $^{***}P < 0.0001$, compared with the control group. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.0001$, compared with the indicated group.

present study, activation of the JNK pathway appears to play an important role in curcumin-induced macrophage apoptosis.

Notably, low concentrations of curcumin (10 and 20 μM) exert protective effect against P19-induced viability inhibition and cytokine production in macrophages. The protective effect of curcumin may be mediated by inhibition of the JNK pathway, since blockade of JNK but not p38 enhances the protective effect of curcumin. However, more data are needed to clarify the protective effects of curcumin. It is reported that P19 triggered TLR2 activation, followed by JNK activation [6]. In contrast, curcumin could reduce the gene expression of TLR2 and inhibit its function in human monocytes and neutrophils [13]. Thus, the TLR-JNK activation induced by P19 and the resulting macrophage apoptosis could be antagonized by curcumin.

In conclusion, our data suggest that both P19 and curcumin inhibit the cell viability of human macrophages. Low doses of curcumin reduce P19-induced macrophage apoptosis. The JNK pathway, but not the p38 or ERK pathway, plays an important role in the protective effect of curcumin against P19 induced macrophage apoptosis. Our study may shed some light on revealing the

mechanism of the protective effect of curcumin against P19 induced macrophages apoptosis, and suggest that curcumin may be a potential candidate in anti-tuberculosis therapy.

Conflict of interest

None.

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