The protective effect of curcumin against the 19-kDa *Mycobacterium tuberculosis* protein-induced inflammation and apoptosis in human macrophages

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Abstract. The *Mycobacterium tuberculosis* 19-kDa lipoprotein (P19) is both cell wall-associated and secreted and is a candidate virulence factor that could cause the apoptosis of human macrophages infected with *M. tuberculosis*. P19 induces TLR2 activation, resulting in the upregulation of death receptors and ligands, followed by a death-receptor signaling cascade. The mechanisms by which P19 induces macrophage apoptosis are not fully characterized. Curcumin, a natural polyphenol, exhibits a variety of pharmacological effects such as antioxidant, anti-inflammatory and antitumor properties. In the present study, we investigated the effect of curcumin on P19-induced apoptosis in human macrophage cells and the underlying mechanisms. The results showed that both P19 and curcumin inhibit the growth of macrophages in a dose- and time-dependent manner. A low dose of curcumin (10 or 20 μM) attenuated both the macrophage cell growth inhibition and the increase in the expression of IL-6 and TNF-α induced by P19. Curcumin also decreased the phosphorylation of JNK and p38 that were induced by P19. However, JNK but not p38 inhibitors reversed the effect of P19 on the growth inhibition of macrophages. These data suggest that curcumin may protect macrophages from P19-induced cell apoptosis via a TLR2-mediated JNK-dependent pathway.

Introduction

The World Health Organization (WHO) estimates that one third of the population worldwide is infected with *Mycobacterium tuberculosis*, and that one tenth of the infected population will develop active tuberculosis (1). Phagocytic cells, such as macrophages, dendritic cells (DCs) and neutrophils, are among the first cells to recognize *M. tuberculosis* within the host. Macrophages and DCs recognize *M. tuberculosis* by pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) (2,3). TLR2, 4 and 9 can mediate the *in vitro* recognition of *M. tuberculosis*. In their absence (particularly of TLR2), the cytokine response of macrophages or DCs to *M. tuberculosis* will be reduced (4-6). The apoptosis of macrophages in *M. tuberculosis* infection is considered to play an important role in the pathogenesis of tuberculosis. However, the mechanism underlying this apoptosis remains unclear.

Lipoproteins, which are post-translationally modified proteins, are generally considered to be potent immune modulators. Mycobacteria are rich in lipoproteins: there are ~100 open reading frames that possess the characteristic amino-terminal acylation motif of lipoproteins in the *M. tuberculosis* genome (7). The *M. tuberculosis* 19-kDa lipoprotein (P19) is cell wall-associated and immunodominant, and has been shown to stimulate the proliferation of CD4^{+} T cells and the release of interleukin 2 (IL-2), interferon-γ (IFN-γ) and IL-12 (8,9). In addition, P19 also acts as an adhesin, interacting with the mannose receptor, and promotes the phagocytosis of mycobacteria (10). Moreover, P19 plays a critical role in inducing the death of macrophages through both extrinsic (TLR2-dependent apoptotic pathway) and intrinsic (mitochondrial pathway) mechanisms (11).

Although the mechanism of apoptosis of mycobacteria-infected macrophages remains to be elucidated, it is clear that the activation of inflammation and apoptosis is involved in this process. Curcumin, a widely used dietary pigment and spice, possesses potent antioxidant, anti-inflammatory and antitumor activities (12,13). Synthetic curcumin analogs and chalcone derivatives are considered as active compounds against *M. tuberculosis*, but their mechanism of action is not fully understood (14,15). Curcumin can suppress cell growth by inhibiting various kinases, such as protein kinase C (PKC), c-Jun NH$_2$-terminal kinase (JNK), and the epidermal growth factor (EGF) receptor kinase (16-18). Curcumin can also inhibit TLR2 gene expression and protein functions possibly via an oxidative process (19). Overall, the mechanism of the
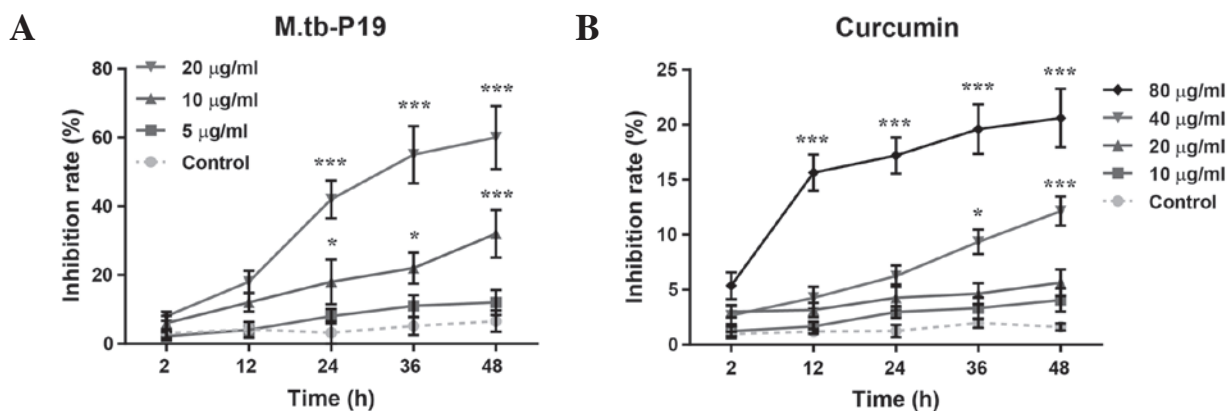
Figure 1. The 19-kDa lipoprotein (P19) and curcumin decrease the cell viability of WBC 264-9C macrophages. Cells were treated for 48 h with different concentrations of (A) *Mycobacterium tuberculosis* (M.tb)-derived P19 or (B) curcumin. The cell viability was measured by the MTT assay. Data are expressed as the mean ± SEM (n=3). *P<0.05, ***P<0.0001, compared to the vehicle-treated (control) group.

anti-tuberculosis effect of curcumin remains unclear and needs to be further elucidated.

In the present study, we tested the hypothesis that curcumin exerts a protective effect against P19-induced inflammation and apoptosis in human macrophages by inhibiting the TLR2-induced and mitogen-activated protein kinase (MAPK)-dependent signaling pathways. Our results demonstrated that both P19 and curcumin induce the apoptosis of macrophages. Furthermore, our study indicated that low doses of curcumin can inhibit the apoptosis induced by P19 in macrophages by activating the JNK pathway.

Materials and methods

Reagents and antibodies. Curcumin was purchased from Sigma-Aldrich (St. Louis, MO, USA), was dissolved in dimethylsulfoxide (DMSO), and stored at -20°C. The final concentration of DMSO in all experiments was <0.1%. The JNK inhibitor SP600125 and the p38 inhibitor SB203580 were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Cell culture reagents, medium, fetal bovine serum (FBS), L-glutamine, and antibiotics were purchased from Thermo Fisher Scientific (Gibco®; Waltham, MA, USA). The antibodies targeting total extracellular signal-regulated kinase (ERK), phospho-ERK (Thr202/Tyr204), total JNK, phospho-JNK (Thr183/Tyr185), total p38, phospho-p38 (Thr180/Tyr182), TLR2 and GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA).

Bacterial strain and P19 isolation. The *M. tuberculosis* strain H37Rv was obtained from the American Type Culture Collection (ATCC 5618; Manassas, VA, USA) in lyophilized form, was reconstituted and used as previously described (20). *M. tuberculosis* 19-kDa lipoprotein (P19) was purified as previously described (21). Briefly, cell-wall fractions were obtained by sonication of resuspended *M. tuberculosis* H37Rv in ice-cold water (20 kHz, 5 cycles, 5 min each). Forty micrograms of protein were dissolved in a reducing sample buffer that contained 0.05 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 1% glycerol, 10% 2-mercaptoethanol, and 0.5 mM/ml Tris-HCl (pH 6.8), were heated for 5 min at 95°C and were subjected to 12% SDS-polyacrylamide gel electrophoresis. Next, proteins
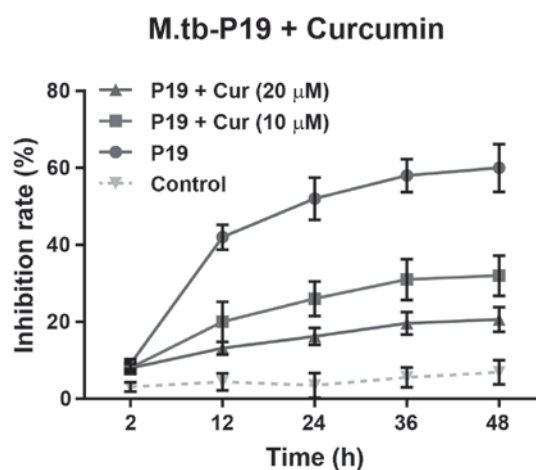

Figure 2. Low concentrations of curcumin (Cur) exert a protective effect against the 19-kDa lipoprotein (P19)-induced decrease in macrophage cell viability. Human WBC 264-9C macrophages were exposed to 20 µg/ml *Mycobacterium tuberculosis* (M.tb)-derived P19 alone or combined with 10 or 20 µM curcumin for 48 h. The cell viability was measured by the MTT assay. Data are expressed as the mean ± SEM (n=3). Control, cells cultured in normal medium.

were transferred onto a nitrocellulose membrane and stained with Ponceau Red to identify the 19-kDa band; this band was also identified using a rabbit monoclonal antibody IT-19 targeting the 19-kDA *M. tuberculosis* antigen (TB Research Material and Vaccine Testing Contract, Colarado State University, Colarado, USA). 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 saline (PBS, pH 7.4) and stored at -20°C. The concentration of the protein was determined with the Bradford method (Bio-Rad, Hercules, USA).

Cell cultures. The WBC 264-9C macrophage line (ATCC HB-8902) was cultivated in RPMI-1640 medium supplemented with 15% FBS, 10 mM HEPES (2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid), 2 mM L-glutamine, and 50 µg/ml gentamicin, at 37°C in a humidified incubator containing 5% CO_2. For P19 treatment, WBC 264-9C cells were placed in 12-well plates with glass coverslips at a density
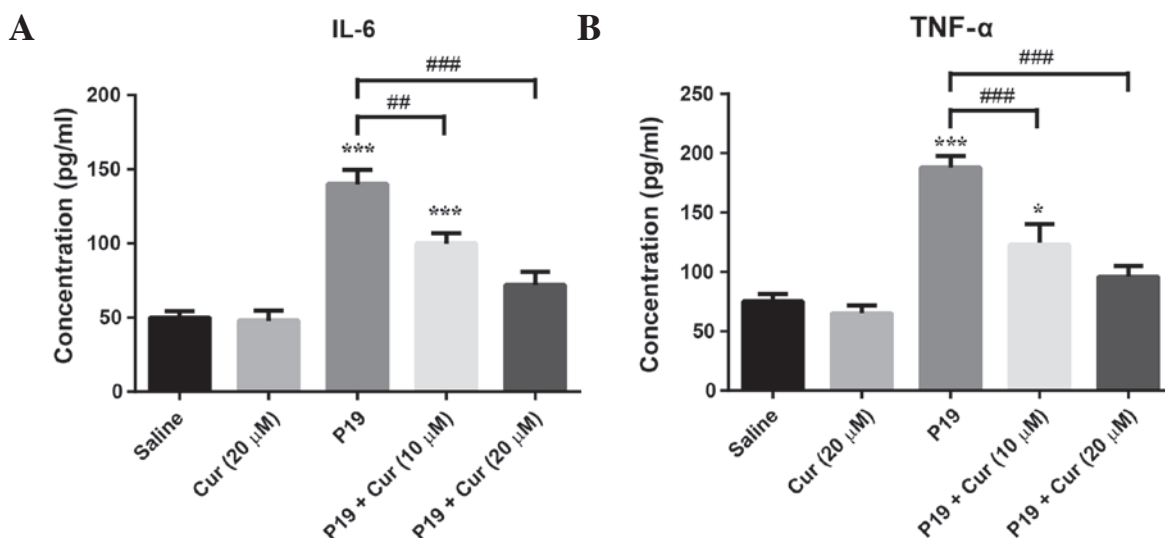
Figure 3. Curcumin (Cur) attenuates 19-kDa lipoprotein (P19)-induced cytokine production in the macrophages. Following treatment with 20 µg/ml P19 alone or combined with 10 or 20 µM curcumin for 48 h, the levels of (A) interleukin 6 (IL-6) and (B) tumor necrosis factor-α (TNF-α) were measured with the enzyme-linked immunosorbent assay. Data are expressed as the mean ± SEM (n=3). *P<0.05, ***P<0.0001, compared to the saline-treated group; ##P<0.01, ###P<0.0001, compared to the indicated group.

of $5x10^5$ cells/ml for 24 h. Cells were then washed and incubated for an additional 18 h in the medium supplemented with 0.1% FBS. The macrophage monolayers in the tissue culture plates were washed with pre-warmed RPMI-1640 medium, which was then replaced with 1 ml of RPMI-1640 medium supplemented with 10 mM HEPES and 0.4% human serum albumin. The cells in the controls groups were cultured in the same RPMI-1640 medium and environmental conditions mentioned above, but were treated with 10% DMSO vehicles later.

Cell viability assays. The effect of P19 and curcumin on the viability of macrophages were evaluated using the MTT assay. Briefly, WBC 264-9C macrophages ($1x10^4$ cells/well) were seeded into a 96-well culture plate. Following adherence, the cells were treated with various concentrations of P19 (5, 10 or 20 µg/ml) or curcumin (10, 20, 40 and 80 µM), JNK inhibitor SP600125 (30 µM) or p38 inhibitor SB203580P38 (20 µM), and incubated at 37°C for various time periods. Then the medium was removed, and 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) 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 Trading AG, Männedorf, Switzerland).

Cytokine assays. The concentrations of IL-6 and tumor necrosis factor-α (TNF-α) in the supernatant of the cell cultures were determined by using Invitrogen™ enzyme-linked immunosorbent assay (ELISA) kits (Thermo Fisher Scientific) for the corresponding human proteins following the manufacturer's instructions.

Western blotting. Following treatment with P19 and/or curcumin, macrophages were rinsed with pre-warmed PBS and lysed in an ice-cold extraction buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1 mM dithiothreitol, and a protease inhibitor

cocktail). The homogenate was incubated on ice for 20 min and centrifuged at 13,000 x g for 20 min at 4°C. Next, the supernatant was collected, and the concentration of the protein in the supernatant was quantified using the Bradford method. The whole-cell lysate from the macrophages was subjected to 12% SDS-PAGE, and subsequently blotted onto a nitrocellulose membrane. The membrane was then incubated with the specific antibodies, using GAPDH as an internal control. Quantification of the protein bands was performed by densitometry using the QuantityOne software (Bio-Rad).

Statistical analysis. The data were expressed as mean ± SEM from three independent experiments, and were analyzed using the SAS 8.2 software package (SAS Institute Inc., Cary, NC, USA). One-way analysis of variance (ANOVA) followed by Bonferroni correction was used to compare the means of three or more groups in the analyses of cell survival, cytokine production and protein expression. The phosphorylation levels of ERK, JNK and p38 were expressed as phosphorylated protein/total protein. P<0.05 was considered to indicate statistically significant differences.

Results

P19 and curcumin decrease the cell viability of WBC 264-9C macrophages. The MTT assay was used to measure the viability of macrophages exposed to P19 or curcumin for 48 h (Fig. 1A). P19 inhibited the cell viability of WBC 264-9C macrophages in a concentration- and time-dependent manner ($F_{concentration (3, 100)} = 55.0$, P<0.0001; $F_{time (4, 100)} = 18.6$, P<0.0001; $F_{interaction (12, 100)} = 4.87$, P<0.0001). Post-hoc analysis revealed that P19 significantly inhibits macrophage viability (10 µg/ml inhibition rate, 32.4%, P<0.0001; 20 µg/ml inhibition rate, 61.5%, P<0.0001). Similarly, curcumin also inhibited macrophage viability (Fig. 1B) in an concentration- and time-dependent manner ($F_{concentration (4, 125)} = 123$, P<0.0001; $F_{time (4, 125)} = 21.8$, P<0.0001; $F_{interaction (16, 125)} = 4.86$, P<0.0001). In WBC 264-9C
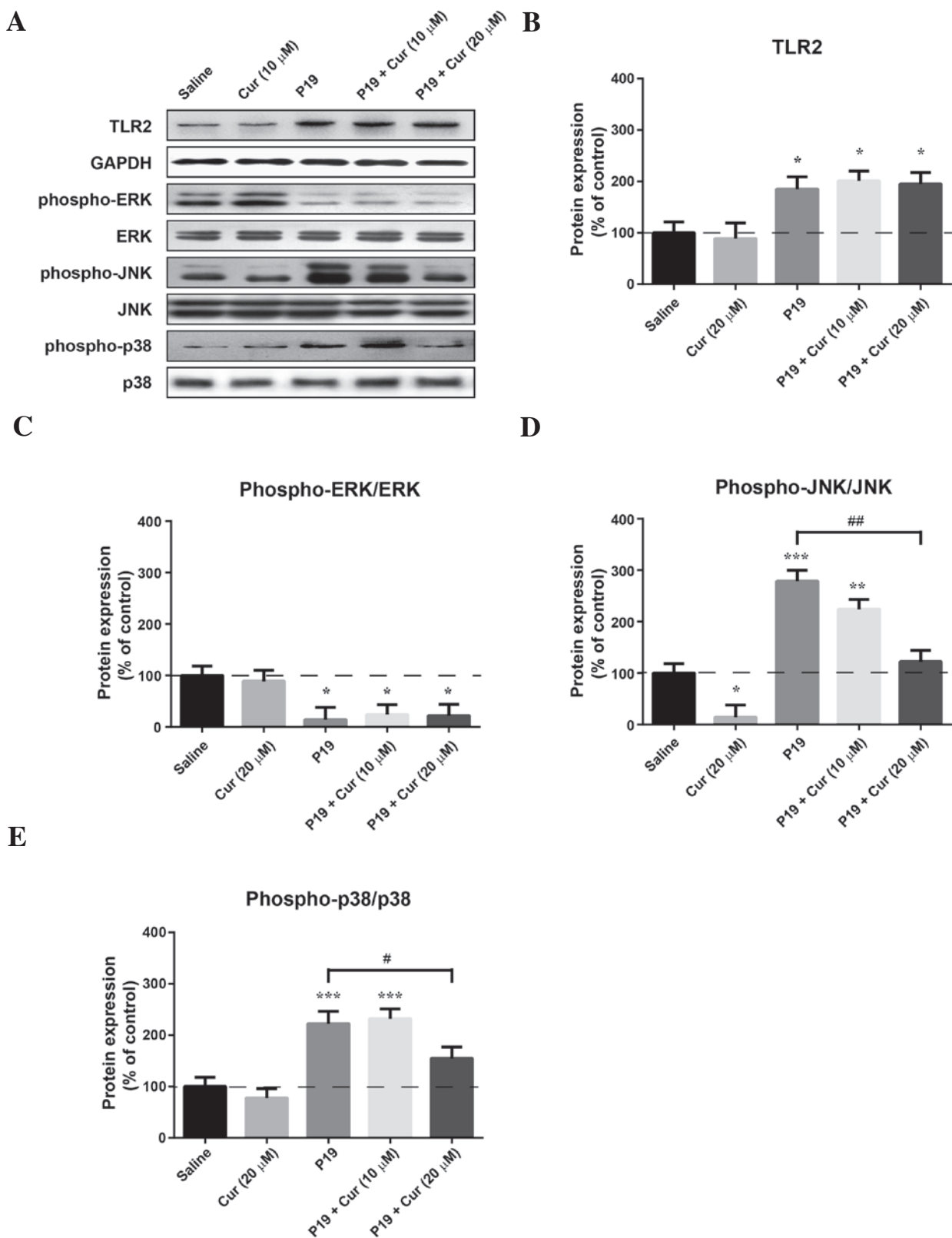
Figure 4. Curcumin (Cur) attenuates 19-kDa lipoprotein (P19)-induced phosphorylation of c-Jun NH$_2$-terminal kinase (JNK) and p38. Human WBC 264-9C macrophages were exposed to vehicle (saline) treatment as a control, or 20 μg/ml P19 treatment alone or combined with 10 or 20 μM curcumin for 48 h. (A) Representative western blots, with GAPDH serving as the loading control. (B) The protein level of TLR2 and the relative phosphorylation levels of (C) extracellular signal-regulated kinase (ERK), (D) JNK and (E) p38 were determined by densitometry analysis. Data are expressed as the mean ± SEM (n=3). *P<0.05, **P<0.01, ***P<0.0001, compared to the saline-treated group; #P<0.05, ##P<0.01, compared to the indicated group. TLR2, toll-like receptor 2.

macrophages, high concentrations of curcumin resulted in 12.1-20.6% inhibition of cell viability (40 μM, P<0.0001 and 80 μM, P<0.0001, respectively). However, low concentrations of curcumin (10 and 20 μM) did not cause a significant inhibition of cell viability. Therefore, 20 μg/ml P19, and 10 and 20 μM curcumin were chosen for the following experiments.
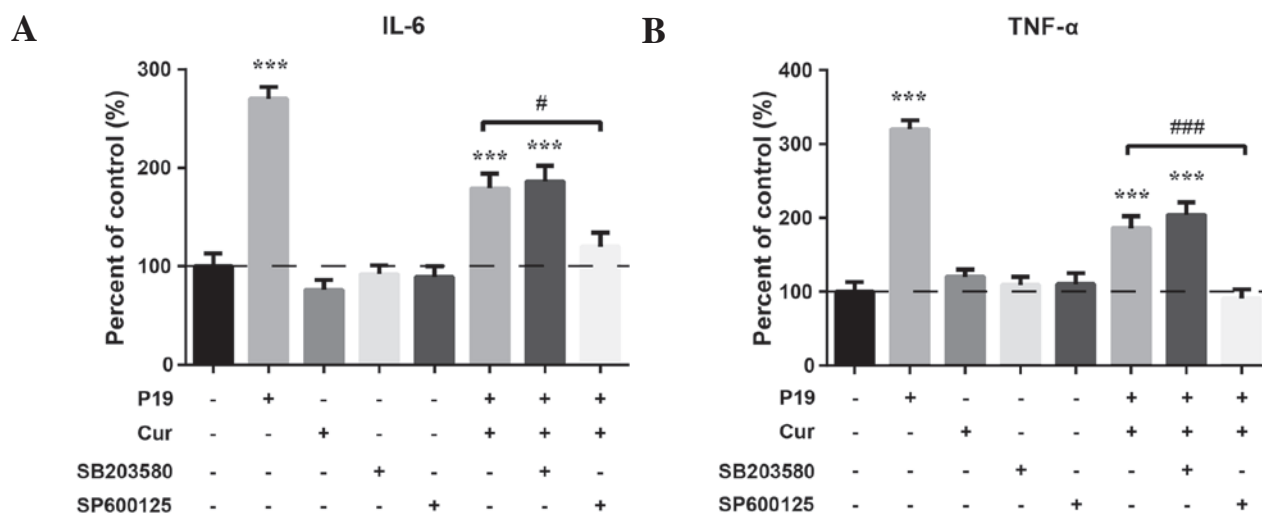
Figure 5. Pharmacological inhibition of c-Jun NH_2-terminal kinase (JNK), but not p38, enhances the protective effect of curcumin (Cur) against 19-kDa lipoprotein (P19)-induced inflammatory responses. Human WBC 264-9C macrophages were treated for 1 h with and without the JNK inhibitor SB203580 (20 μM) or the p38 inhibitor SP600125 (30 μM) prior to treatment with 20 μg/ml P19 and/or 20 μM curcumin for 48 h. The levels of (A) interleukin 6 (IL-6) and (B) tumor necrosis factor-α (TNF-α) were measured with the enzyme-linked immunosorbent assay. Data are expressed as the mean ± SEM (n=3). ***$P<0.0001$, compared to the saline-treated (control) group. #$P<0.05$, ###$P<0.0001$, compared to the indicated group.

Low concentrations of curcumin exert a protective effect against P19-induced inhibition of viability in macrophages. Human macrophages were exposed to vehicle (saline) treatment as a control, or to 20 μg/ml P19 alone or combined with 10 or 20 μM curcumin for 48 h. As expected, P19 treatment alone markedly reduced the cell viability (58.7%, $P<0.0001$) (Fig. 2). Notably, the combined treatment with P19 and a low concentration of curcumin (10 μM) attenuated the inhibition of cell viability (32.2%, $P<0.0001$), compared to P19 treatment alone (Fig. 2). A higher concentration of curcumin (20 μM) further decreased (20.6%, $P<0.0001$) the inhibition in cell viability induced by P19.

Curcumin attenuates P19-induced cytokine production in macrophages. ELISA was used to measure the IL-6 and TNF-α levels in the macrophages treated with 20 μg/ml P19 alone or combined with 10 or 20 μM curcumin for 48 h (Fig. 3). The IL-6 and TNF-α levels were not significantly affected by treatment with 20 μM curcumin alone. As expected, IL-6 and TNF-α levels were significantly elevated upon P19 treatment ($P<0.0001$), whereas the combination of 10 μM curcumin and P19 significantly decreased the P19-induced IL-6 and TNF-α production (IL-6, $P<0.01$; TNF-α, $P<0.0001$). Strikingly, treatment with 20 μM curcumin completely offset the P19-induced cytokine production.

Curcumin attenuates P19-induced phosphorylation of JNK and p38. To investigate the mechanism underlying the protective effects of curcumin against the P19-induced inflammatory responses, the expression of TLR2 and the phosphorylation of ERK, JNK and p38 were measured by western blot analysis (Fig. 4A). The expression of TLR2 did not significantly change following treatment with 20 μM curcumin (Fig. 4B), whereas treatment with 20 μg/ml P19 significantly increased the TLR2 level ($P<0.05$). The P19-induced increase in the TLR2 level was not affected by cotreatment with 10 or 20 μM curcumin.

As shown in Fig. 4, P19 decreased the phospho-ERK level ($P<0.05$), and increased the phospho-JNK ($P<0.0001$) and the phospho-p38 ($P<0.0001$) levels. Although treatment with 20 μM curcumin alone had no significant effect on the p38 basal level, its phosphorylation was significantly decreased relative to treatment with P19 upon treatment with 20 μM curcumin ($P<0.05$). Furthermore, the phosphorylation of JNK was decreased by both curcumin treatment alone ($P<0.05$) and when curcumin was combined with P19.

Pharmacological inhibition of JNK, but not p38, enhances the protective effect of curcumin against P19-induced inflammatory responses. To determine the role of JNK and p38 phosphorylation in P19-induced inflammatory responses, we pre-treated the macrophages with 20 μM SP600125 (the JNK-specific inhibitor) or 30 μM SB203580 (the p38-specific inhibitor) for 1 h prior to treatment with 20 μM curcumin and 20 μg/ml P19. Cytokine production was determined by ELISA. Fig. 5 shows that treatment with the JNK inhibitor significantly decreased IL-6 ($P<0.05$) and TNF-α ($P<0.01$) levels, compared to the combination of curcumin and P19. IL-6 and TNF-α levels were slightly reduced after treatment with the p38 inhibitor, but the difference was not significant. 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.

Discussion

P19 plays an important role in the apoptosis of macrophages during *M. tuberculosis* infection. Induction of macrophage apoptosis by *M. tuberculosis* and thus, evasion from host defense, has been proposed as one of the mechanisms promoting *M. tuberculosis* virulence. Recently, the contribution of P19 in human *M. tuberculosis* infections received increasing attention. Elucidating the mechanism underlying P19-induced macrophage death during *M. tuberculosis* infections is important for the development of new anti-tuberculosis strategies.

Furthermore, polyphenols such as curcumin possess numerous desirable qualities for anti-tuberculosis therapy.

Synthetic bacterial lipoproteins have been reported to induce macrophage apoptosis through TLR2, which implies that the mycobacterial 19-kDa lipoprotein (P19) may be a *M. tuberculosis* apoptosis-inducing factor. P19 is an abundantly expressed, cell wall-associated and secreted glycolipoprotein, with a highly conserved six-residue consensus sequence for lipidation adjacent to the hydrophobic signal peptide. Lopez *et al* showed that P19 induces apoptosis in the human monocytic cell line THP-1 and in human monocyte-derived macrophages (22). Consistently, our data showed that P19 induces the apoptosis of macrophages in a time- and dose-dependent manner. The apoptosis-inducing effect of P19 may be TLR2-dependent, since pre-treatment with blocking anti-TLR2 antibody was previously shown to inhibit the P19-induced apoptosis in THP-1 cells or TLR2-expressing Chinese hamster ovary cells (22). Recently, Sanchez *et al* revealed a novel mechanism for the *M. tuberculosis*-induced death of macrophages, whereby P19 triggers an intrinsic or mitochondrial pathway, associated with cytochrome *C* and the apoptosis-inducing mitochondrial factor (11). Overall, P19 may induce macrophage apoptosis.

Curcumin has been known to inhibit the proliferation and/or induce cell death in a number of human cancer cell lines (23-27). Interestingly, curcumin is non-cytotoxic to healthy 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 exceeding 10 mg/day without any side-effects (28). The present study provided evidence that curcumin can induce the death of macrophages in a dose- and time-dependent manner. Although the fact that curcumin induces apoptosis is well established, the exact mechanism underlying this effect is not yet fully elucidated. Potential mechanisms of curcumin-induced apoptosis include inhibition of various protein kinases such as PKC, JNK, and the EGF receptor kinase. Curcumin can also activate apoptotic pathways, such as p53-mediated apoptosis, in human basal cell carcinoma cells under stress conditions (29). Mitochondrial dysfunction triggered by enhanced Bax expression is also involved in curcumin-induced apoptosis (30,31). Moreover, curcumin can block the nuclear factor-κB (NF-κB) cell survival pathway (32,33). However, the mechanism underlying curcumin-induced apoptosis has been mainly revealed from tumor cell lines, whereas the mechanism underlying curcumin-induced apoptosis in macrophages still remains unclear.

We found that low concentrations of curcumin (10 and 20 μM) exert a protective effect against P19-induced apoptosis in macrophages, and attenuate P19-induced cytokine production. The protective effect may be mediated by JNK inhibition, since JNK, but not p38 inhibitors enhanced the protective effect of curcumin. Although additional data are needed to clarify the protective effect of curcumin against P19-induced apoptosis, we can conclude that curcumin and P19 may play opposing roles in inducing macrophage apoptosis. TLR2 receptor-induced JNK activation is one of the candidate signaling pathways involved in P19-induced apoptosis. In human monocytes and neutrophils, curcumin treatment decreased the gene expression of *TLR2* and impaired the function of the protein (19). By contrast, P19 was shown to induce TLR2 activation, followed by JNK activation (11,34).

In summary, we demonstrated that both P19 and high doses of curcumin can decrease the viability of human macrophages. Low doses of curcumin reduce the P19-induced apoptosis and the increased cytokine levels in the macrophages. The protective effect of curcumin may be attributed to its ability to block the TLR2-mediated JNK activation induced by P19. Our study has shed some light on the mechanism underlying the protective effect of curcumin against P19-induced macrophage apoptosis, and suggests that curcumin may be used as a therapeutic agent for the treatment of tuberculosis.

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