# Vitamin C inhibits apoptosis in THP-1 cells in response to incubation with *Mycobacterium tuberculosis*

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Abstract. Tuberculosis (TB) is a chronic and fatal zoonotic infectious disease caused by Mycobacterium tuberculosis (M. tb) infection. The THP-1 cell line is a cell model for studying the function, mechanism and signaling pathways of macrophages; macrophages are the primary host cells of *M. tb.* Macrophages are important for the progression of tuberculosis, as they affect the release of various inflammatory cytokines, including IL-1β, IL-6 and TNF-α. Vitamin C is a trace element for the human body. Its biological efficacy depends on its redox abilities and its role as a cofactor in several enzymatic reactions. However, whether vitamin C can protect THP-1 cells from *M. tb* infection has not yet been reported. The present study aimed to further investigate the effects of vitamin C on M. tb infection-induced THP-1 cell injury and its mechanism. In the present study, MTT assay, reverse transcription-quantitative PCR, EdU cell proliferation assay, western blotting, immunohistochemistry, flow cytometry and TUNEL staining assays were used to assess the cell viability, inflammation and apoptotic levels of THP-1 cells induced by M. tb following vitamin C treatment. The effect of vitamin C on M. tb infection was also assessed using Balb/c mice; pulmonary injury was assessed by H&E staining of the lung tissue. The results demonstrated that vitamin C markedly

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attenuated cellular damage caused by *M. tb* infection. The results demonstrated that vitamin C reduced the expression of *M. tb*-induced apoptosis-related proteins (Cleaved-caspase-9, Cleaved-caspase-3, Bcl-2, Cyt-c) and inflammatory factors (IL-1 $\beta$ , IL-6, NLRP3, TNF- $\alpha$ , IL-8, NF- $\kappa$ B) in THP-1 cells and reduced apoptosis. Overall, these results suggested that vitamin C may reduce lung damage caused by *M. tb* infection.

### Introduction

Mycobacterium tuberculosis (M. tb) is the pathogen that causes tuberculosis (1). It can invade all organs of the body and is the most common cause of tuberculosis. Globally, each year, ~10 million individuals become infected with M. tb (2), resulting in ~1.7 million mortalities, which poses a serious threat to public health (3). Therefore, identifying a novel theoretical and experimental basis for treating tuberculosis is important. THP-1 cells are widely used in the study of monocyte and macrophage-related mechanisms and signaling pathways. THP-1 cells are easy to cultivate and expand in the laboratory, have a more stable genetic background, and do not possess the problem of individual differences associated with peripheral blood mononuclear cells, which is conducive to the reproduction of experimental results (4). Therefore, THP-1 cells are an ideal tool for studying immunity and inflammation.

Alveolar macrophages are not only the primary site for M. tb colonization and reproduction (5), but they are also the first line of defense against M. tb infection. Several reports indicate that M. tb can induce necrosis (6), apoptosis (7) and autophagy in macrophages (8). Furthermore, M. tb-infected macrophages can survive in the host cell for a substantial period of time (9). Previous studies have indicated that vitamin C is the most important water-soluble antioxidant in human plasma and mammalian cells (10,11), suggesting that vitamin C may also have important cellular functions. It is well-established that vitamin C can prevent the occurrence of scurvy and protect healthy cells from oxidative damage (12). The self-protective mechanism of *M*. *tb* in cells generates free radicals, which increases cellular toxicity (13). Therefore, additional research is required to determine whether antioxidants are beneficial to patients with an M. tb infection. As a

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scavenger of free radicals, it remains unclear whether vitamin C affects THP-1 cells infected with *M. tb*.

To address this, after incubating THP-1 cells with M. tb, the apoptotic signaling and cellular inflammatory factors of the host cells following vitamin C treatment were further studied. Animal experiments were performed to verify the protective effect of vitamin C after M. tb infection.

#### Materials and methods

Cell culture. THP-1 cells (American Type Culture Collection), a model commonly used for studying the function of macrophages (14), were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). The cells were cultured in a 5% CO<sub>2</sub> incubator at 37°C before seeding them in a 6-well plate at a density of  $2x10^6$  cells/well or  $1x10^6$  cells/well; 2x10<sup>6</sup> cells/well for western blotting, RT-qPCR, EdU cell proliferation experiments and 1x106 cells/well for flow cytometry and TUNEL assay experiments. The cells were treated with phorbol 12-myristate 13-acetate (PMA; 100 ng/ml) (Thermo Fisher Scientific, Inc.) at 37°C for 24 h. PMA transformed THP-1 cells into adherent macrophages and then the cells were incubated with M. tb (MOI:10; Chinese Center for Disease Control and Prevention; CCDC, Beijing, China) and vitamin C (150 µM/ml) at 37°C for 24 h. The concentrations and treatment durations were used for all cell assays.

*MTT assay*. An MTT assay was used to assess the viability of cells. THP-1 cells were seeded in a 96-well plate at a density of  $5x10^3$  cells/well, and PMA was used to induce cell transformation before being pretreated with various concentrations (0, 50, 100, 150, 200 and 300  $\mu$ M/ml) of vitamin C for 24 h at 37°C. Subsequently, cells were treated with lipopolysaccharide (LPS; 1  $\mu$ g/ml) (Thermo Fisher Scientific, Inc.) for 12 h at 37°C, and MTT solution (20  $\mu$ l) was added to each well according to the manufacturer's instructions. The cells were then incubated for 4 h at 37°C. Finally, DMSO (100  $\mu$ l) was added to each well, and the absorbance (560 nm) was measured using a microplate reader (Bio-Rad Laboratories, Inc.).

Western blotting. Total protein was extracted from the cells in the different treatment groups [i) Control (untreated) group; ii) M. tb group; iii) vitamin C group; and iv) M. tb + vitamin C group] using a M-PER mammalian protein extraction reagent kit (Thermo Fisher Scientific, Inc.). A BCA protein assay kit was used (Thermo Fisher Scientific, Inc.) to measure the total protein concentration. Subsequently, 10  $\mu$ g protein was mixed with 6X loading buffer (Takara Bio, Inc.) and loaded on 10% SDS-PAGE, followed by transfer to PVDF membranes. After blocking with SuperBlock (Thermo Fisher Scientific, Inc.) 37°C for 1 h, the membrane was incubated overnight at 4°C with the primary antibodies, including GAPDH (cat. no. 5174), Cleaved-caspase-9 (cat. no. 20750), Cleaved-caspase-3 (cat. no. 9661), Bcl-2 (cat. no. 15071), cytochrome c (Cyt-c; cat. no. 12963), Bax (cat. no. 5023), IL-1ß (cat. no. 12703), IL-6 (cat. no. 12912) or NLR family pyrin domain-containing 3 (NLRP3; cat. no. 15101) (all 1:1,000; Cell Signaling Technology, Inc.). The samples were then incubated with the secondary antibodies HRP-conjugated Affinipure goat anti-mouse IgG (H+L) (1:10,000; cat. no. SA00001-1; ProteinTech Group, Inc.) or HRP-conjugated Affinipure goat anti-rabbit IgG (H+L) (1:10,000; cat. no. SA00001-2; ProteinTech Group, Inc.) at room temperature for 1 h. A ECL chemiluminescence kit (Analytik Jena US LLC) was used to visualize protein bands. The intensity of each protein band was normalized to the respective GAPDH band and analyzed using ImageJ (version 1.46; National Institutes of Health).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from THP-1 cells using the MiniBEST Universal RNA extraction kit (Takara Bio, Inc.), and RNA was reverse-transcribed to cDNA using a PrimeScript<sup>™</sup> RT Reagent kit (Takara Bio, Inc.). The following temperature protocol was used for reverse transcription: 37°C for 15 min and 85°C for 5 sec. Subsequently, the obtained cDNA was amplified with qPCR on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a TB Green Fast qPCR Mix kit (Takara Bio, Inc.). The following thermocycling qPCR conditions were used for amplification: 95°C for 30 sec; followed by 40 cycles of 95°C for 5 sec and 65°C for 30 sec. Relative expression levels were evaluated using the  $2^{-\Delta\Delta Cq}$ method and normalized to GAPDH as the internal control (15). qPCR reactions were performed using the following primers: GAPDH forward, 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse, 5'-GGCTGTTGTCATACTTCTCATGG-3'; TNF-α forward, 5'-CCTCTCTCTAATCAGCCCTCTG-3' and reverse, 5'-GAGGACCTGGGAGTAGATGAG-3'; and IL-8 forward, 5'-TTTTGCCAAGGAGTGCTAAAGA-3' and reverse, 5'-AACCCTCTGCACCCAGTTTTC-3'.

*EdU cell proliferation assay.* Cell proliferation was performed using an EdU cell proliferation kit (cat. no. C0075S; Beyotime Institute of Biotechnology). The cells (2x10<sup>6</sup> cells/well) were seeded into 6-well plates and incubated at 37°C for 24 h then the cells of different treatment groups were incubated with *M.tb* (MOI:10) and vitamin C (150  $\mu$ M/ml) at 37°C for 24 h. THP-1 cells were then incubated with EdU (10  $\mu$ mol/l) for 2 h at 37°C, washed three times with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.3% Triton X-100 for 15 min at room temperature. Hoechst was used to stain the nuclei for 10 min at room temperature after the incubation with 0.3% Triton X-100. Cells were washed three times with PBS and the images were obtained using a fluorescence microscope.

Flow cytometry. The cells  $(1x10^6 \text{ cells/well})$  were seeded into 6-well plates and incubated at 37°C for 24 h, then the cells of different treatment groups were incubated with *M.tb* (MOI:10) and vitamin C (150  $\mu$ M/ml) at 37°C for 24 h. After treatment, THP-1 cells were then washed thrice in cold PBS and pelleted by centrifugation (5 min; 650 x g; 37°C). Next, the supernatant was discarded, and the pellet was resuspended in 100  $\mu$ l 1X Annexin V-binding buffer (Invitrogen; Thermo Fisher Scientific, Inc.), to which 5  $\mu$ l Alexa Fluor<sup>®</sup> 488 Annexin V-FITC (Component A) and 1  $\mu$ l 100  $\mu$ g/ml PI working solution were added and further incubated at room temperature for 15 min. After the incubation period, cells were analyzed using a FACSCanto II cytometer (BD Biosciences) and CytExpert 2.0 software (Beckman Coulter, Inc.) to determine the rate of apoptosis in early + late stages of cells.

*TUNEL assay.* The cells  $(1x10^6 \text{ cells/well})$  were seeded on coverslips in 6-well plates and incubated at 37°C for 24 h, then the cells of different treatment groups were incubated with *M.tb* (MOI:10) and vitamin C (150  $\mu$ M/ml) at 37°C for 24 h. After treatment, THP-1 cells were then washed thrice in PBS and fixed with 4% paraformaldehyde (at room temperature for 15 min), followed by permeabilization using 0.2% Triton X-100 (37°C for 10 min). Next, THP-1 cells were incubated with a TUNEL reaction mixture at 37°C for 60 min (Thermo Fisher Scientific, Inc.), washed thrice with PBS, and counterstained with DAPI for 15 min at 37°C (Thermo Fisher Scientific, Inc.). Finally, 80 cells for each group were observed under a confocal laser scanning microscope and processed on Leica Confocal Software v.2.6.1 (Leica Microsystems GmbH).

Animal experiments. A total of 16 male BALB/c mice (6-8 weeks old; weight,  $20\pm25$  g) were purchased from Jackson ImmunoResearch Laboratories, Inc. All animal experiments and protocols were approved by the Ethical Committee of Ningxia University (Yinchuan, China; specific pathogen free grade; approval no. 2020-024). All animals were housed in a pathogen-free facility (22±2°C; 50±5% humidity) with a 12-h light/dark cycle, and the mice had ad libitum access to food and water. After 1 week of acclimation, the mice were randomly divided into four treatment groups: i) Control group (50  $\mu$ l of 0.9% normal saline administered intragastrically); ii) M. tb group [50  $\mu$ l M. tb (50  $\mu$ g/ml) by intraperitoneal injection]; iii) vitamin C group [50  $\mu$ l vitamin C (0.5  $\mu$ M/ml) administered intragastrically]; and iv) M.tb + vitamin C group  $[50 \,\mu l \, vitamin \, C \, (0.5 \,\mu M/ml)$  administered intragastrically and M. tb (50  $\mu$ g/ml) by intraperitoneal injection]. Treatments were intragastrically administered once a day for 21 days. Rapid weight loss >15-20% without significant signs of dehydration and weakness was defined as a potential humane endpoint for the present study.

*Histological evaluation*. Mice were sacrificed after 21 days by cervical dislocation. The absence of heartbeat and respiration were used as criteria to confirm mouse death. Fresh lung tissue was isolated and washed once with PBS. The tissue was fixed with 4% paraformaldehyde for 20 min at 37°C and then embedded in paraffin. The embedded tissue was cut into  $4-\mu$ m thick sections. Finally, the tissue was stained with hematoxylin and eosin at 37°C for 10 min. The images of stained tissues were then scored by three independent pathologists who were blinded prior to scoring as follows: 1, Normal; 2, slight tissue damage; 3, mild tissue damage; and 4, severe tissue damage (confocal laser scanning microscopy; magnification, x100).

Immunohistochemistry. Mice were sacrificed after 21 days. Fresh lung tissue was isolated, fixed overnight with 4% paraformaldehyde (Solarbio, P1110) at 4°C. Then the samples were dehydrated in ethanol and xylene (70% ethanol 30 min; 80% ethanol 30 min; 95% ethanol 30 min; 100% ethanol 30 min twice; xylene 30 min twice), embedded in paraffin and cut into sections as aforementioned  $4-\mu M$  thick sections. Lung tissues was transferred to 3-aminopropyl-triethoxysilane-treated

microscope slides (ZLI-9001; Zhongshan Company) for immunofluorescence staining. In brief, sections were deparaffinized and rehydrated (xylene 5 min twice; 100% ethanol 3 min twice; 90% ethanol 3 min; 80% ethanol 3 min; 70% ethanol 3 min). After three washes in distilled water, antigen retrieval was performed by microwaving for 15 min in 0.01% sodium citrate buffer (pH 6.0). After three washes in PBS, the lung tissues were treated with 3% H<sub>2</sub>O<sub>2</sub> in PBS for 20 min to quench endogenous peroxidase activity. Nonspecific binding was blocked with 5% BSA in PBS for 15 min at room temperature. Sections were then incubated with primary antibody (NF- $\kappa$ B; 1:200; cat. no. 8242; Cell Signaling Technology, Inc.) overnight at 4°C. After three washes in PBS, the lung tissue sections were incubated for 20 min at room temperature with horseradish peroxidase-labeled goat anti-rabbit IgG (Zhongshan Company), and rinsed with PBS. The antibody complex was detected using DAB reagent (Zhongshan Company). Finally, the sections were incubated with hematoxylin staining solution (Zhongshan Company) for 20 sec at room temperature and washed once with distilled water. Images were captured using a light microscope (magnification, x100).

Statistical analysis. All data collected were obtained from at least three independent experiments for each condition. All results were analyzed using GraphPad Prism version 6.0 (GraphPad Software, Inc.) and are presented as the mean  $\pm$  standard deviation (unless otherwise shown). Unpaired Student's t-test was used to compare differences between two groups, and one-way ANOVA followed by Tukey's post hoc test was used to compare differences between >2 groups. The statistical analysis of the histological score results was conducted using Kruskal-Wallis test followed by Dunn's post hoc test and are presented as median + interquartile range. P<0.05 was considered to indicate a statistically significant difference.

#### Results

M. tb infection induces the expression of apoptosis-related proteins, and vitamin C pretreatment increases the viability of THP-1 cells after LPS stimulation. After THP-1 cells were incubated with M. tb for 24 h, the protein expression levels of the apoptosis-related proteins cleaved caspase-9, cleaved caspase-3, Bcl-2 and Cyt-c were analyzed using western blotting (Fig. 1A). Cleaved caspase-9, cleaved caspase-3 and Cyt-c levels were significantly increased in the *M*. tb infection group compared with the control group (Fig. 1A and B). Furthermore, incubation with *M*. *tb* significantly suppressed the protein expression levels of Bcl-2 (Fig. 1A and B). An MTT assay was used to determine the effects of LPS stimulation on the viability of THP-1 cells after vitamin C pretreatment. THP-1 cells were pretreated with different concentrations of vitamin C (0, 50, 100, 150, 200 and 300  $\mu$ M/ml) for 24 h and then treated with LPS (1  $\mu$ g/ml) for 12 h (Fig. 1C). Experimental results demonstrated that vitamin C pretreatment significantly increased the viability of THP-1 cells after LPS stimulation in a dose-dependent manner. These results suggested that M. tb infection promoted apoptosis and that vitamin C pretreatment increased the viability of THP-1 cells. Subsequently, it was assessed whether vitamin C affected THP-1 cell viability infected with M. tb.



Figure 1. Effect of vitamin C on the viability of THP-1 cells during incubation with *M. tb* and stimulation by LPS. (A) After incubating THP-1 cells with *M. tb* for 24 h, the protein expression levels of apoptosis-related proteins, including Cleaved caspase-9, Cleaved caspase-3, Bcl-2 and Cyt-*c*, were analyzed using western blotting. (B) Cleaved caspase-9, Cleaved caspase-3, Bcl-2 and Cyt-*c* protein semi-quantitative expression levels. GAPDH was used as a loading control. (C) THP-1 cells were pretreated with different concentrations of vitamin C (0, 50, 100, 150, 200 or 300  $\mu$ M/ml) for 24 h and then treated with LPS for 12 h. Cell viability was measured using an MTT assay. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. control; *M. tb, Mycobacterium tuberculosis*; Cyt-*c*, cytochrome *c*; LPS, lipopolysaccharide.

Vitamin C increases the viability and reduces the levels of inflammatory factors released by THP-1 cells following M. tb infection. Vitamin C exerted a protective effect on the viability of THP-1 cells after LPS stimulation. Thus, whether vitamin C could inhibit the levels of inflammatory factors in THP-1 cells after *M. tb* infection was assessed. To determine whether vitamin C regulated the inflammatory response of THP-1 cells after M. tb infection, the protein levels of inflammatory factors IL-1β, IL-6 and NLRP3 were assessed using western blotting (Fig. 2A). The results indicated that the expression levels of IL-1β, IL-6 and NLRP3 were significantly higher in the *M*. *tb* group compared with the control group and that vitamin C treatment significantly decreased their expression levels compared with the M. tb group (Fig. 2A-D). Furthermore, TNF-a and IL-8 mRNA expression levels were significantly lower in the vitamin C + M. tb group compared with the *M*. tb group (Fig. 2E and F). Therefore, western blotting and RT-qPCR analysis demonstrated that vitamin C exerted a significant anti-inflammatory effect. However, the anti-inflammatory effect of vitamin C should be studied further in animal models. IL-1 $\beta$  and IL-6 ultimately regulate inflammatory gene expression by activation of NF- $\kappa$ B (16). Given the important role of NF- $\kappa$ B in host defense and its role in regulating inflammation, the expression of NF- $\kappa$ B in lung tissues was examined using immunohistochemistry. As

presented in Fig. 2G, compared with the control group, the M. tb group exhibited higher expression levels of NF- $\kappa$ B, and the expression of NF- $\kappa$ B in the vitamin C + M. tb was markedly lower. These results suggested that vitamin C may attenuate M. tb infection by inhibiting the inflammatory response.

Vitamin C reduces apoptosis of THP-1 cells after M. tb infection. To determine whether vitamin C regulated apoptosis of THP-1 cells after M. tb infection, western blotting was used to analyze the protein levels of apoptosis-related factors, including cleaved caspase-3, Bax, cleaved caspase-9 and Cyt-c (Fig. 3A). Compared with the M. tb group, treatment with vitamin C significantly decreased the protein expression levels of cleaved caspase-3, Bax, cleaved caspase-9 and Cyt-c following M. tb infection (Fig. 3C). An EdU cell proliferation assay was used to investigate the effect of vitamin C on THP-1 cell proliferation after M. tb infection. As presented in Fig. 3B, red fluorescence, indicative of proliferation, was markedly reduced by M. tb treatment and increased by vitamin C. Moreover, apoptosis was evaluated using flow cytometry and TUNEL staining assays. Flow cytometry results demonstrated that M. tb infection significantly promoted apoptosis of THP-1 cells compared with the control, and the apoptotic rate was  $\sim$ 74% in the *M*. *tb* group. By contrast, vitamin C treatment following M. tb treatment decreased the apoptotic rate as



Figure 2. Effect of *M. tb*, vitamin C (100  $\mu$ M/ml) and the combination of both on THP-1 cell inflammation. (A) After *M. tb*, vitamin C and vitamin C + *M. tb* treatment for 24 h, the protein expression levels of the inflammatory factor markers IL-1 $\beta$ , IL-6 and NLRP3 were analyzed using western blotting. Semi-quantitative protein expression levels of (B) IL-1 $\beta$ , (C) IL-6 and (D) NLRP3. The mRNA expression levels of (E) TNF- $\alpha$  and (F) IL-8 were measured using reverse transcription-quantitative PCR. (G) Representative immunohistochemical staining images of NF- $\kappa$ B in lung tissues. Scale bar, 50  $\mu$ m. \*\*P<0.01 and \*\*\*P<0.001 vs. control; \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. *M. tb. M. tb, Mycobacterium tuberculosis*; NLRP3, NLR family pyrin domain-containing 3.

compared with the *M*. *tb* group and the apoptotic rate was only ~48% (Fig. 3D and E). TUNEL staining assay results demonstrated that the number of TUNEL-positive cells was increased in the *M*. *tb* group. By contrast, in the vitamin C

and vitamin C + M. tb groups, the number of TUNEL-positive cells was markedly reduced (Fig. 3F). These results suggested that treatment with vitamin C may attenuate M. tb infection by inhibiting apoptosis in THP-1 cells.



Figure 3. Effect of *M. tb*, vitamin C (100  $\mu$ M/ml) and the combination of both on THP-1 cell apoptosis. (A) After *M. tb*, vitamin C and vitamin C + *M. tb* treatment for 24 h, the protein expression levels of the apoptosis factor markers Cleaved caspase-3, Bax, Cleaved caspase-9 and Cyt-*c* were analyzed using western blotting. (B) Representative images of EdU staining in the control, *M. tb*, vitamin C and vitamin C + *M. tb* groups. EdU labelling is shown in red, and nuclei are labelled by Hoechst staining in blue (magnification, x100). (C) Cleaved caspase-3, Bax, Cleaved caspase-9 and Cyt-*c* protein semi-quantitative expression levels. GAPDH was used as a loading control. (D) Cell apoptosis was detected by Alexa<sup>®</sup> Fluor 488 Annexin V and PI assay. (E) Apoptotic rate (%) in the control, *M. tb*, vitamin C and vitamin C + *M. tb* groups. TUNEL labelling is shown in green, and nuclei are labelled using DAPI (blue). Scale bar, 50  $\mu$ M. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. control; \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. *M. tb*. *M. tb*. *Mycobacterium tuberculosis*; Cyt-*c*, cytochrome *c*.

Vitamin C reduces lung damage after M. tb infection. Finally, to confirm the immunosuppressive function of M. tb and the protective effect of vitamin C, mice were treated with

vitamin C and M. tb intragastrically, hematoxylin and eosin staining was performed and the pathological changes in the lung tissue were observed under a microscope. The lung tissue



Figure 4. Morphological changes of hematoxylin and eosin-stained lung tissues following vitamin C treatment observed in BALB/c mice infected with *M. tb.*. The morphological changes in lung tissues were observed using hematoxylin and eosin staining in the different treatment groups. Black arrows indicate necrotic tissue; green arrows indicate inflammatory cell infiltration; red arrows indicate interstitial edema. Representative images of the morphological changes in lung tissues in the (A) control group, (B) *M. tb* group, (C) vitamin C group and (D) vitamin C + *M. tb* group. (E) Corresponding pathological scoring. Scale bar, 50  $\mu$ m, \*P<0.05 vs. *M. tb. M. tb*, *Mycobacterium tuberculosis*.

sections from the control group (Fig. 4A) and the vitamin C group (Fig. 4C) exhibited a clear alveolar structure with no edema, congestion or inflammatory cell infiltration in the interstitial space. In the *M*. *tb* group (Fig. 4B), lung tissue sections were notably damaged, with interstitial edema, congestion and inflammatory cell infiltration, indicating a pathological appearance. Compared with the *M*. *tb* group, the degree of alveolar wall damage and infiltrating inflammatory cells in the vitamin C + M. *tb* group were also significantly decreased (Fig. 4D and E).

## Discussion

The pathogenic mechanism of M. tb has always been the focus of tuberculosis research (17). According to WHO statistics, the death rate of pulmonary tuberculosis is 1/10,000. Due to the relatively effective anti-tuberculosis drug treatment currently available, the mortality rate of pulmonary tuberculosis is significantly lower than that before drug treatment (18). Although the rate of mortalities caused by tuberculosis has declined, it remains one of the most serious threats to global public health (19). Therefore, exploring a novel method to study the immunomodulatory effect of M. tb in the process of alveolar macrophage infection and the immune escape mechanism of pathogens has become a subject of current research.

Vitamin C is an important element in the body and plays a notable role in the immune system. Previous studies have revealed that vitamin C can improve immunity by generating (20) and activating immune cells (21) and resisting cell damage caused by pathogens and free radicals (22). The possible mechanism of action of vitamin C as an antioxidant is by inhibiting the production of reactive oxygen species (ROS) (23), and it is also a powerful regulator of  $Ca^{2+}$ signaling (24). Therefore, vitamin C may alter intracellular  $Ca^{2+}$  homeostasis and affect apoptosis. However, the ability of vitamin C to protect THP-1 cells infected by *M*. *tb* may help to understand the pathological mechanism of tuberculosis.

Inflammation is a basic pathological process, corresponding to the body's defense response to the stimulation of various pro-inflammatory factors (25). THP-1 cells can secrete cytokines, such as TNF-a, IL-2, IL-3, granulocyte macrophage colony-stimulating factor and NLRP3, amongst others. IL-8, also known as C-X-C motif chemokine ligand 8, is a cytokine secreted by macrophages and epithelial cells (26). Chemokines/inflammatory factors play an important role in the cellular immune response (27). Pro-inflammatory cytokines are needed for the coordination of cell-mediated immune responses (28), often regulating the growth, activation, differentiation and homing of immune cells to the site of infection, aiming to control and eradicate intracellular pathogens (29). Therefore, these pro-inflammatory cytokines have been identified as molecular targets for inflammation control (30). The present study demonstrated that treatment with *M*. *tb* significantly increased the levels of inflammatory factors in cells. It was concluded that vitamin C may attenuate *M. tb* infection by inhibiting inflammation.

Apoptosis is a cellular mechanism of programmed cell death that is tightly regulated by a family of proteases called caspases (31), which are normally present in healthy cells as inactive precursors, but become active during apoptosis (32). Apoptosis plays a notable role in cell survival under certain stress conditions by scavenging proteins and damaged organelles to maintain cell homeostasis and integrity (33). The major effector caspases, caspase 3 and 9 (34), are the key molecules in the intrinsic pathway of apoptosis (35). Caspase-3 is present in the cell as an inactive dimer, which is cleaved and activated by Caspase-9 (36). Different proteases cleave the caspase-3 zymogen to activate it (Cleaved Caspase-3). Cleaved Caspase-3 further cleaves different substrates, leading to the expansion of the protease cascade and eventually cell death (37). The cleaved substrates lead to alterations in protein function and thus cellular changes associated with apoptosis. Therefore, caspase-3 activation leads to induction of cellular apoptosis (38).

Bcl-2 is the founding member of the Bcl-2 family of apoptosis regulatory proteins that either induce (pro-apoptotic) or inhibit (anti-apoptotic) apoptosis (39). The anti-apoptotic Bcl-2 is classified as an oncogene, as damage to the Bcl-2 gene has been shown to cause a number of types of cancer, including lymphoma (40). Bcl-2 inhibits apoptosis by preventing the release of Cyt-c from the mitochondria to the cytoplasm (41). Cyt-c activated in response to apoptotic stimuli and alter the permeability of the mitochondrial outer membrane, which is considered a key step in apoptosis (42).

Cyt-*c* possesses multiple functions, including the generation and scavenging of ROS, and it plays an important role in the mitochondrial electron transport chain (43). In addition, Cyt-*c* is a key regulator of apoptosis. In the current study, THP-1 cells were incubated with *M. tb* for 24 h with vitamin C or vitamin C + M. tb. First, it was demonstrated that *M. tb* treatment significantly decreased expression of the anti-apoptotic protein Bcl-2. The protein expression levels of cleaved caspase-3, Bax, cleaved caspase-9 and Cyt-*c* in the vitamin C + M. tb groups were significantly decreased compared with the *M. tb* group. Next, an EdU cell proliferation assay was used to assess cell proliferation, and the results indicated that red fluorescence representing proliferation was inhibited by *M. tb* treatment and was promoted by vitamin C. These results supported the hypothesis that vitamin C decreased apoptosis and promoted cell proliferation in THP-1 cells infected with *M. tb*.

In conclusion, the present study demonstrated that the molecular mechanism of vitamin C promoted apoptosis in THP-1 cells infected by M. tb. However, the precise mechanism by which vitamin C promotes the expression of apoptosis-related proteins needs further research and it may also be necessary to repeat the experiments in a different type of macrophage cell line to further test the hypothesis. Vitamin C may reduce the damage to mitochondrial function caused by M. tb infection by inhibiting the release of apoptosis-related proteins and inflammatory factors. The results indicated that the mechanism of action of vitamin C may enhances the signal transduction in the host cell to inhibit phagosome and lysosome fusion, eventually inhibiting apoptotic signaling pathways present in macrophages to decrease the occurrence of apoptosis.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### **Authors' contributions**

YuW designed the project, revised the article and provided technical guidance. DX designed the project, revised the article and coordinated all aspects of the present work. FS and YiW participated in all experiments, performed data analysis, created the figures and wrote the article. XL was responsible for sample preparation and documentation. YiW and FS confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

All animal experiments and experimental protocols were approved by the Ethics Committee of Ningxia University (Yinchuan, China; approval no. 2020-024).

### Patient consent for publication

Not applicable.

#### **Competing interests**

The authors declare that they have no competing interests.

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