

Original Article

The regulatory effect of the NF- κ B signaling pathway on macrophage autophagy after *M. tuberculosis* infection

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Abstract: Macrophages can eliminate MTB pathogens by regulating autophagy and inhibiting their proliferation in host cells. The NF- κ B signaling pathway involves several biological behaviors. Whether the NF- κ B signaling pathway regulates macrophage autophagy after *M. tuberculosis* infection remains unclear. Macrophage RAW264.7 cells were cultured and divided into a control group (infected with BCG (Bovis Bacillus Calmette-Guérin), an NF- κ B activation group which was supplemented with 5 μ M TNF- α to activate NF- κ B, and a NF- κ B inhibition group (treated with 5 μ M BAY 11-7082) followed by an analysis of the time of autophagy and the multiplicity of the infection of the macrophages, Atg5 and Atg12 expressions using real time PCR, the expressions of LC3, P62, NF- κ B, and mTOR using Western blot, and the IL-6 and IL-1 β secretions using ELISA. The macrophages were infected for 16 h, and the multiplicity of the infections was 12, and significant autophagy was observed. The NF- κ B activation group showed significantly increased NF- κ B and mTOR expressions, decreased LC3, Atg5, and Atg12 expressions, up-regulated P62, and increased IL-6 and IL-1 β secretions ($P < 0.05$). The NF- κ B inhibitors significantly inhibited the NF- κ B and mTOR expressions, increased the LC3, Atg5, and Atg12 expressions, down-regulated P62, and decreased the secretions of IL-6 and IL-1 β ($P < 0.05$). The activation of NF- κ B signaling promotes mTOR expression and inflammation, down-regulates proteins which are required for autophagosome membrane formation, and inhibits autophagy in macrophages.

Keywords: NF- κ B signaling pathway, *Mycobacterium tuberculosis*, macrophage, autophagy, mTOR

Introduction

Tuberculosis (TB) is characterized as *Mycobacterium tuberculosis* (MTB) infection [1]. Because tuberculosis is contagious and refractory, and cause serious damage to health, it poses a serious threat to human health [2]. Although tuberculosis has been curbed to some extent due to advances in medical technology, the tuberculosis rate is increasing due to environmental pollution, a reduction in people's immunity, the use of immunosuppressive drugs, the number of immunodeficiency patients, and the spread of AIDS [3, 4]. Moreover, increased population mobility and tuberculosis's drug resistance cause an increased transmission probability and difficulty in treatment [5]. At present, the incidence rate is still high in developing and underdeveloped countries, which is a major global health problem and leads to a high mortality rate [5, 6]. Tuberculosis

is occurring in the young and middle-aged populations with an increased incidence rate [7]. Because the onset of tuberculosis is occult with a long incubation period and slow progress, it can easily be missed [8]. Although the main site of the disease is the lungs, *Mycobacterium tuberculosis* can enter the skin, meninges, peritoneum, intestines, and bones along with the blood and lymphatic circulatory systems [9].

Mycobacterium tuberculosis is a typical intracellular parasite. It infects and reproduces mainly in macrophages after infecting the body. Macrophages can be used as the host cells of *Mycobacterium tuberculosis*, thus resisting the body's immune response [10, 11]. MTB colonizes macrophages, and macrophages can eliminate MTB pathogens by regulating autophagy and inhibiting their proliferation in host cells [12]. MTB, by escaping autophagy, proliferates and infects cells. Therefore, one of the

Table 1. Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	ACCAGGTATCTTGTTG	TAACCATGTCAGCGTGGT
Atg5	TCACATCATCGATTAGT	GGTTGGTGTATCTCAT
mTOR	CATTCTTATGCACCATT	TTGGTGGTCTCGTAGTCT
Atg12	AGTTCGCTGGTCTCATG	CAGCGTAGATGCCTCAGT

immune mechanisms of the macrophage defense against MTB is through autophagy [13, 14]. The NF-κB signaling involves a variety of biological behaviors and regulates inflammation, immunity, and autophagy [15, 16]. However, whether NF-κB signaling regulates macrophage autophagy after *M. tuberculosis* infection has not been reported.

Materials and methods

Main instruments and reagents

A human macrophage cell line (THP-1) was preserved in our laboratory and stored in liquid nitrogen. The Bovine *M. tuberculosis* BCG vaccine strain was purchased from Shanghai Biological Products Research Institute Co., Ltd. and kept by our laboratory and stored in liquid nitrogen. BAY 11-7082 was from the APEX BIO Corporation of the United States. TNF-α was purchased from Sigma, USA. The phorbol ester (PMA), the RPMI1640 and DMEM mediums, the fetal bovine serum (FBS), the calf serum (BSA), and the c-chain double antibody were purchased from Gibco, USA, and Sigma, USA. The dimethyl sulfoxide and MTT powder was from Gibco, and the trypsin-EDTA solution was from Sigma, USA. The PVDF membrane was from Pall Life Sciences, the EDTA was purchased from Hyclone, USA, the ECL reagent was purchased from Amersham Biosciences, and the rabbit anti-mouse NF-κB monoclonal antibody, the rabbit anti-Mouse autophagy-related antibody, and the (HRP)-labeled IgG was from Abcam, USA. The RNA extraction and reverse transcription kits were from Axygen, USA. The IL-6 and IL-1β ELISA kits were purchased from Shanghai Lichen Biotechnology Co., Ltd.

THP-1 cell culture, induction, and grouping

The THP-1 cells were cultured in an RPMI1640 medium at 37°C in a 5% CO₂ incubator to maintain the number of cells at 1×10⁸/culture flask. The subcultured THP-1 cells were diluted to 1×10⁶/ml, inoculated into a 35 mm culture dish, and cultured in a serum-free RPMI1640 medium containing 100 ng/ml PMA

and 0.3% BSA to induce differentiation for 72 hours. The experiment used 3rd-8th generation logarithmic growth phase cells. The THP-1 cells were placed into the control group, which was infected with BCG (Bovis Bacillus Calmette-Guerin). After the colony was prepared on a Middlebrook 7H10 solid medium, the monoclonal colonies were picked, ground with Tween warm water, and added to Middlebrook 7H9 liquid medium and cultured. 1×10⁶ THP-1 cells were seeded in a 6-well plate, BCG was added to each cell well, and the ratio of the number of cells to the number of bacteria was 1:10, and the cells were cultured. After establishing the optimal autophagy model, the NF-κB activation and inhibition groups were established. In the NF-κB activation group, after the BCG-infected macrophage autophagy model was established, 5 μM TNF-α was added to activate the NF-κB. In the NF-κB inhibition group, 5 μM BAY 11-7082 was added to inhibit the NF-κB.

Real time PCR quantification of the mTOR, Atg5, and Atg12 expressions

The total RNA was extracted using Trizol reagent, and a DNA reverse transcription synthesis was performed. The primers are shown in **Table 1**. The real-time PCR reaction conditions were 4 minutes after 94°C, 94°C 30 S, 56°C 60 S, 72°C 40 S, for a total of 40 cycles. The data were collected using the PCR reactor software. GAPDH was used as a reference. According to the fluorescence quantification, the starting cycle numbers (CT) of all the samples and standards were calculated. The 2-ΔCt method was used to analyze the gene expressions.

Western blot

The logarithmic growth phase THP-1 cell protein was extracted from each group using a RIPA lysis buffer and then quantified and followed by separation on a 10% SDS-PAGE for western blot using LC3, P62, NF-κB, and GAPDH (1:2000) monoclonal antibodies. The membrane was developed after the addition of chemiluminescence for 1 min.

ELISA analysis of the inflammatory factor secretions

The supernatants of each group were collected to determine the IL-6 and IL-1β secretions using ELISA according to the kit's instructions.

NF-κB in macrophage autophagy

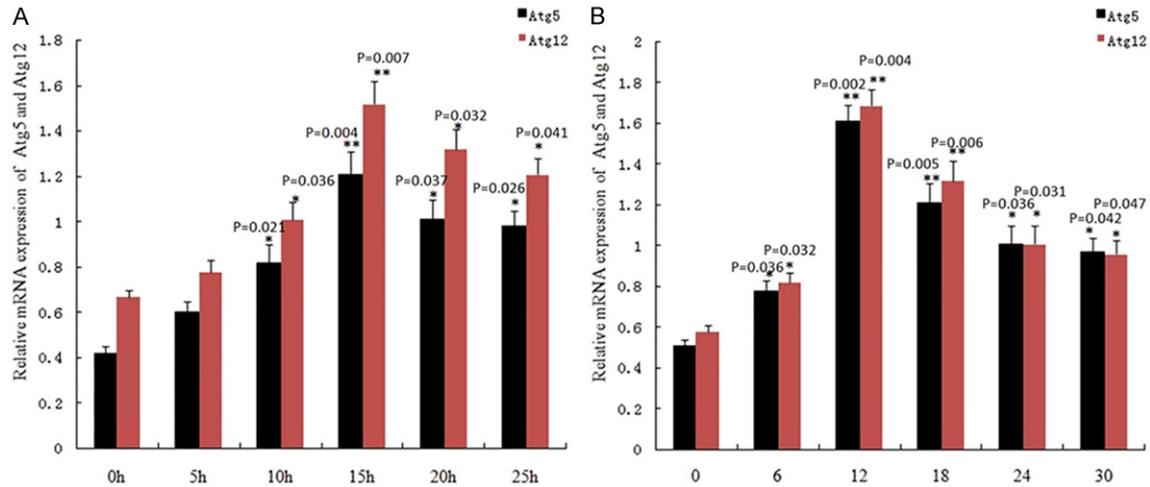


Figure 1. BCG infection induces autophagy in macrophages. A. The effect of different BCG infection times on the expressions of Atg5 and Atg12 in macrophages; B. The effect of different BCG infections on the expressions of Atg5 and Atg12 in macrophages; compared with the control group, *P<0.05; **P<0.01.

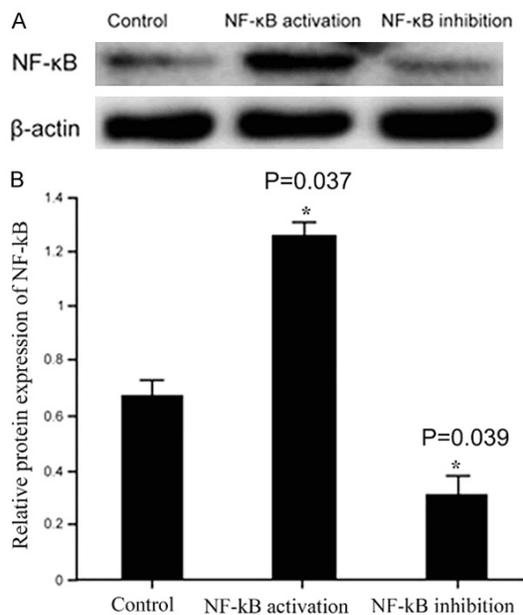


Figure 2. The effect of the NF-κB signaling pathway on the expression of NF-κB in the autophagy of macrophages infected with *Mycobacterium tuberculosis*. A. Western blot analysis of the regulation of the NF-κB signaling pathway on the expression of NF-κB in the autophagy of *Mycobacterium tuberculosis*-infected macrophages; B. A statistical analysis of the effects of NF-κB expression, compared with the control group, *P<0.05.

Statistical analysis

The data were processed using SPSS 19.0 software and displayed as the means ± standard deviations and assessed using one-

way ANOVA. P<0.05 indicated a significant difference.

Results

BCG infection induces autophagy in macrophages

The Atg5 and Atg12 expressions were analyzed using Real time PCR. With prolonged infection times, the expressions of the autophagy genes were increased (P<0.01), with their expressions at 16 h the highest, then followed by a decrease (P<0.01). When the number of infections was 12 (the number of cells), the number of bacteria was 1:12, and the Atg5 and Atg12 expressions reached their highest levels (P<0.01), so the time of the BCG-infected macrophage autophagy was 16 h, and the multiplicity of the infection was 12 (**Figure 1**).

*The effect of the NF-κB signaling on the NF-κB levels in the macrophage autophagy infected by *Mycobacterium tuberculosis**

This study used *M. tuberculosis* to induce autophagy in macrophages. A western blot analysis found that the NF-κB level in the macrophage autophagy induced by *Mycobacterium tuberculosis* in the NF-κB activation group was significantly elevated compared to the control group (P<0.05). The NF-κB inhibitors significantly inhibited the expression of NF-κB in the macrophages induced by *Mycobacterium tuberculosis* infection (P<0.05) (**Figure 2**).

NF-κB in macrophage autophagy

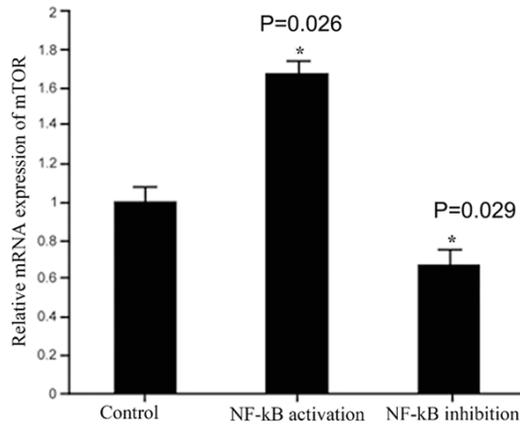


Figure 3. The effect of the NF-κB signaling pathway on the mTOR expression in the autophagy of *Mycobacterium tuberculosis*-infected macrophages. Compared with the control group, *P<0.05.

The effect of the NF-κB signaling pathway on the mTOR expressions in the autophagy of Mycobacterium tuberculosis-infected macrophages

Our real-time PCR analysis of the effect of the NF-κB signaling pathway on the mTOR expression in the autophagy of *Mycobacterium tuberculosis* infection-induced macrophages showed that the expression of mTOR in the autophagy of macrophages infected by *Mycobacterium tuberculosis* in the NF-κB activation group was significantly increased (P<0.05). The NF-κB inhibitors significantly inhibited the expression of mTOR in the macrophage autophagy induced by the *Mycobacterium tuberculosis* infection (P<0.05) (**Figure 3**).

The effects of the NF-κB signaling pathway on the autophagy-related genes in the Mycobacterium tuberculosis-infected macrophages

A real-time PCR analysis demonstrated that the Atg5 and Atg12 induced by *Mycobacterium tuberculosis* in the NF-κB-activated group was significantly downregulated (P<0.05), but it was significantly increased in the *Mycobacterium tuberculosis*-infected NF-κB-inhibited group (P<0.05) (**Figure 4**).

The effect of the NF-κB signaling pathway on the autophagy-associated proteins induced by Mycobacterium tuberculosis induced by macrophages

A western blot analysis of the effects of the NF-κB signaling pathway on the autophagy-

associated proteins LC3 and P62 of *Mycobacterium tuberculosis* infection found a significantly-reduced LC3 level and an increased P62 level in the NF-κB-activated group (P<0.05). In the NF-κB inhibition group, *Mycobacterium tuberculosis* infection significantly promoted the LC3 level and reduced the P62 level (P<0.05) (**Figure 5**).

The effect of the NF-κB signaling pathway on the inflammatory factors in the autophagy of Mycobacterium tuberculosis infected macrophages

Our ELISA analysis of the effects of the NF-κB signaling pathway on the inflammatory factors in the autophagy of *Mycobacterium tuberculosis* infection-induced macrophages reported significantly elevated IL-6 and IL-1β secretions in the macrophage autophagy induced by *Mycobacterium tuberculosis* in the NF-κB-activated group (P<0.05), which was significantly decreased in the supernatant of the macrophages infected by *Mycobacterium tuberculosis* in the NF-κB inhibition group (P<0.05) (**Figure 6**).

Discussion

Tuberculosis remains a serious problem that threatens people's health. Spinal tuberculosis is a chronic extrapulmonary tubercular disease that progresses slowly and is difficult to diagnose, and it often leads to irreversible nerve damage, including paralysis and causing serious social and economic problems, so its treatment has become a global health priority [17, 18].

After it infects a patient, *Mycobacterium tuberculosis* is engulfed by macrophages, the body's first line of defense. On the one hand, macrophages can be used as immune cells to phagocytose bacteria, but on the other hand, *Mycobacterium tuberculosis* acts as an intracellular parasite that can inhibit macrophage cell death, phagosome lysosomal fusion, and interference with signal transduction mechanisms, and it can resist macrophage apoptosis, which in turn leads to MTB resistance to host cell immune killing [19-21]. As a target cell of MTB, macrophages are the main cells of the body's immune response to MTB. When MTB invades the body, macrophages themselves can mediate inflammation through apoptosis, autophagy, necrosis and the TLR signaling

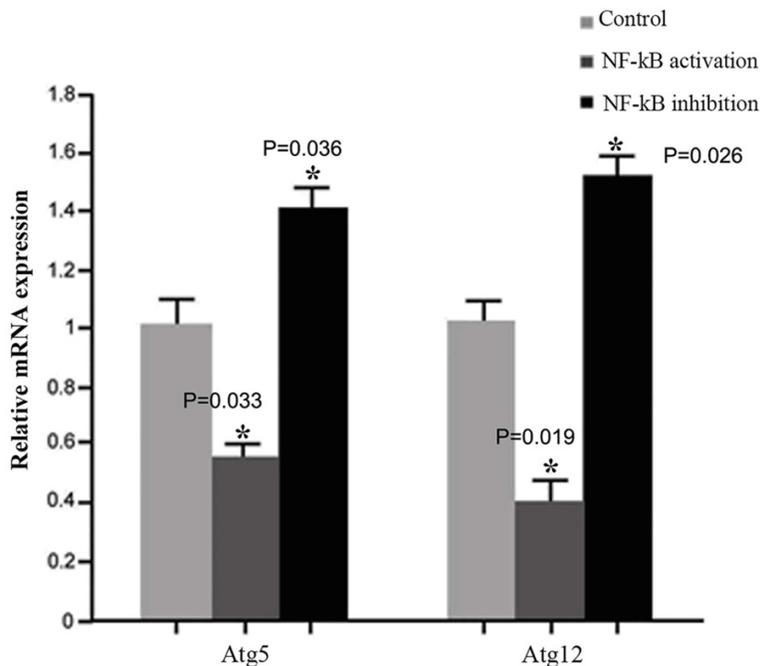


Figure 4. The effects of the NF- κ B signaling pathway on the autophagy-related genes in *Mycobacterium tuberculosis*-infected macrophages. Compared with the control group, *P<0.05.

pathway, which is beneficial in clearing MTB. The defense mechanisms are apoptosis and autophagy, which are beneficial to the survival of pathogens [22, 23]. NF- κ B regulates inflammation and the immune response. The activation of NF- κ B may cause increased secretions of the inflammatory factors. If abnormalities occur in various links, a certain degree of disorder or damage to cell functions may occur [15, 16]. This study first determined the time point and concentration of autophagy in the formation of MTB-infected macrophages. The macrophages were infected for 16 h, and the multiplicity of infection was 12, and obvious autophagy could be detected. Therefore, the NF- κ B agonists and inhibitors were added after the formation of the autophagy was determined. The expressions of NF- κ B and mTOR were increased in the NF- κ B-activated group and the LC3 involved in autophagosome membrane formation was decreased with decreased Atg5 and Atg12, and elevated P62, IL-6, and IL-1 β secretions. The NF- κ B inhibitors inhibited the NF- κ B expression, decreased the mTOR expression, increased the LC3, Atg5, and Atg12 expressions, and down-regulated the P62 expression. This result suggests that

NF- κ B signaling can regulate the secretions of the inflammatory factors and significantly alter the autophagy formation of the MTB-infected macrophages. The mammalian rapamycin protein (mTOR) is a downstream signaling molecule of NF- κ B signaling that regulates cell growth, proliferation, differentiation, and the cell cycle, and can promote the occurrence of autophagy in cells [24, 25]. Our study suggests that the regulation of NF- κ B signaling on the autophagy of MTB-infected macrophages may be closely related to mTOR changes.

Conclusion

NF- κ B signaling can activate mTOR expression, promote inflammation, down-regulate

the protein required for autophagosome membrane formation, and inhibit macrophage autophagy. The inhibition of the NF- κ B signaling pathway inhibits mTOR expression, inhibits inflammation, upregulates proteins required for autophagosome membrane formation, and promotes autophagy in macrophages. Therefore, targeting NF- κ B signaling can regulate the autophagy of macrophages during infection with *Mycobacterium tuberculosis*, and then it can regulate the outcome of the *Mycobacterium tuberculosis* infection.

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Disclosure of conflict of interest

None.

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NF-κB in macrophage autophagy

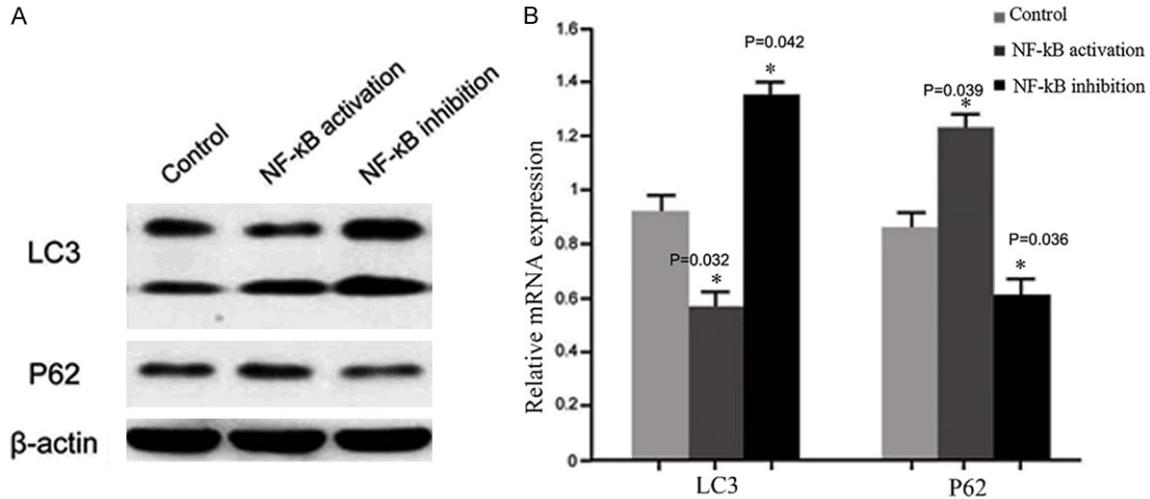


Figure 5. The effect of the NF-κB Signaling Pathway on the autophagy-associated proteins induced by *Mycobacterium tuberculosis*. A. A western blot analysis of the regulation of the NF-κB signaling pathway on the autophagy-associated proteins LC3 and P62 of the *Mycobacterium tuberculosis*-infected macrophages; B. The NF-κB signaling pathway induces macrophage autophagy-associated proteins in *Mycobacterium tuberculosis* infection. A statistical analysis of the effects of LC3 and P62 compared with the control group, *P<0.05.

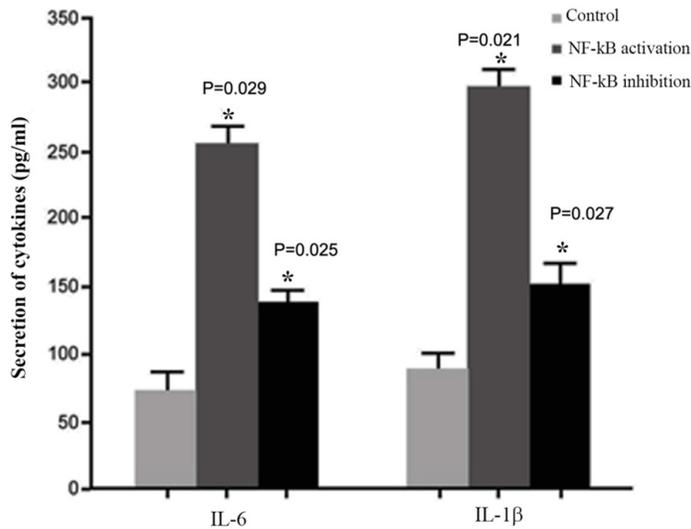


Figure 6. The effect of NF-κB signaling pathway on the inflammatory factors in macrophage autophagy induced by *Mycobacterium tuberculosis* infection. Compared with the control group, *P<0.05.

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