

## Original Article

# Mcl-1 signals pathway inhibitors in mouse peritoneal macrophage apoptosis infected with the Xinjiang strain of *M. tuberculosis*

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**Abstract:** A present, myeloid cell leukemia-1 (Mcl-1) was suggested as a potential new target for controlling latent TB infection. Therefore, we investigated the role of the Mcl-1 signalling pathway in mouse peritoneal macrophages infected with XJ-MTB, aiming at finding a new strategy for TB management in Xinjiang. We using TUNEL, Immunohistochemical analysis, ELISA, HE, RT-PCR and Western blot detected macrophages apoptosis, the damage of mice tissues and the expression of apoptosis genes and proteins. Results found that inhibition of the Mcl-1 signalling pathway not only reduced the survival of intracellular XJ-MTB, but also increased peritoneal macrophage apoptosis in latent XJ-MTB-infected mouse peritoneal macrophages and relieved the pathological damage of mouse organs infected with XJ-MTB, especially MAPK signalling pathway inhibitor PD98059 ( $P < 0.05$ ). Moreover, after inhibitor PD98059 treated mouse peritoneal macrophages infected with XJ-MTB, Bcl-2, Bax and Mcl-1 were reduced, while Cytochrome-c and Caspase-8 protein levels were significantly increased, and Cytochrome-c protein levels was significant higher than Caspase-8 ( $P < 0.05$ ). In conclusion, the MAPK signalling pathway inhibitor PD98059 down-regulated Mcl-1 expression and effectively increased macrophage apoptosis in mice infected with XJ-MTB. Furthermore, it also relief pathological organ damage and promote the elimination of inflammation. The intrinsic apoptotic pathway plays a predominant role in the regulatory role.

**Keywords:** Mcl-1 signalling pathway, MTB infection, macrophages, cell apoptosis

## Introduction

Tuberculosis (TB) is both an old and new disease caused by *Mycobacterium tuberculosis* (MTB). After decades of research, we currently face serious strains of multidrug-resistant TB (MDR-TB), and the effects of prevention and control in the present situation are not ideal [1]. In 2013, an estimated 9 million individuals developed TB, and 1.5 million died from this disease. China alone accounted for 11% of the total cases worldwide [2]. Differences in TB incidence have been observed in China [3-5]. Nevertheless, of all the provincial regions in China, the second highest TB incidence (464/100,000) occurred in the Xinjiang Uygur Autonomous Region (Xinjiang). Therefore, it is imperative to offer the prevention and control

strategy for cases of the Xinjiang strain of *M. tuberculosis* (XJ-MTB) infection.

Macrophages are the major host cells for MTB, and they exert their innate macrophage defence mechanisms via necroptosis, apoptosis and autophagy, which influence the outcome of infection [6-9]. Apoptotic death reduces the viability of different mycobacterial species including MTB. During MTB infection, multiple molecules and their associated signalling pathways in macrophages are involved in the regulation of the cellular fates of host cells. Studies have found that myeloid cell leukemia-1 (Mcl-1) plays a special role in MTB infection [10, 11].

The Mcl-1 gene is an anti-apoptotic member of the B cell lymphoma-2 (Bcl-2) family, which is

the key factor in the upstream regulation of apoptosis [12]. In 2003, Sly et al. [11] found that when MTB exhibits long-term survival in macrophages, Mcl-1 gene expression is high in infected macrophages, and macrophages simultaneously show a decline in their apoptotic rate. In 2016, Wang et al. [13, 14] found that Mcl-1 expression intervention effectively promotes TB infection-related host macrophage apoptosis, proving that Mcl-1 plays an important role in the regulation of macrophage apoptosis in MTB.

Currently, the expression of Mcl-1 is regulated through three signalling pathways, primarily through the activation of Janus kinase/signal transducer and activator of transcription (JAK/STAT), mitogen-activated protein kinase (MAPK), and phosphatidylinositol-3 kinase (PI-3K) pathways [15-17]. The JAK/STAT signalling pathway includes a variety of biological responses, such as cell proliferation, apoptosis, and immune system regulation. STAT3 is the most common member of the STAT family [18]. The JAK/STAT signalling pathway inhibitor AG490 can block JAK2/STAT3 phosphorylation, activated and caused by a variety of cytokines, and then block the JAK/STAT signal transduction pathway. Numerous experiments have confirmed that AG490 promotes the apoptosis of tumour cells with almost no side effects on normal cells [19, 20]. The MAPK pathway is clearly the extracellular signal regulating kinase of the MAPK/ERK pathway in the present study [21] as well as in the phosphorylation of the ERK1/2 pathways, which are involved in cell proliferation, differentiation and cell cycle regulation [22]. The PI-3K signalling pathways was plays a role in its resistance to apoptosis by influencing a variety of effector molecules downstream [23]. In human macrophages, the expression level of Mcl-1 showed a significant reduction after the application of the inhibitor LY294002, which blocks the PI-3K pathway [24].

However, our experimental group previous studies confirmed that the suppression of Mcl-1 expression enhances peritoneal macrophage apoptosis in mice infected with MTB. Therefore, the current experiment analyses this phenomenon from the perspective of morphology to test whether inhibiting the Mcl-1 expression signalling pathway can effectively relieves the pathological damage of mouse organs infected with XJ-MTB. Furthermore, we explored the reg-

ulatory mechanism of Mcl-1 by detecting the key genes and proteins of the apoptotic pathways. We hope that these findings can provide a new strategy to prevent and control XJ-MTB.

### Materials and methods

#### *Animals*

Female healthy BALB/c mice (6-8 wk old, weighing 18±20 g), and purchased from the Experimental Animal Centre of Shihezi University (Xinjiang, China). The mice were subsisted under a 12 h light/darkness cycles, and clear and enough foods and water were provided ad libitum. All experiments in this study were implemented according to the protocols and principle approved by the Institutional Animal Ethics Committee (IAEC) prior to experimentation.

#### *Bacteria*

The advantage of XJ-MTB was identified early and saved by the laboratory. The bacteria was used to a mixture of Sutong culture and normal saline (0.5 ml; volume ratio of 3:1), and 100- $\mu$ L bacterial dilutions were spread on modified Roche medium. After 22 weeks, we selected the bacteria that grew well on the Roche medium, after re-suspending it, mixed them with a small amount of normal saline solution containing 0.05% Tween-80 (Tianjin, China). The bacterial suspension was adjusted to the McFarland standard corresponding to  $1 \times 10^7$  CFU/ml. Aliquot of the stock were stored at -80°C freezer.

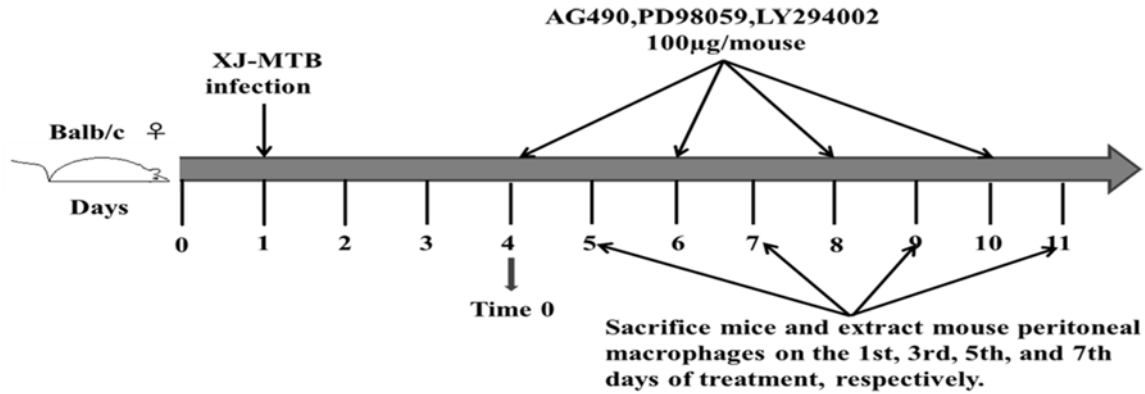
#### *Inhibitors*

The inhibitors AG490, PD98059, and LY294002 were obtained from Sigma (St Louis, MO, USA). Dilution method: AG490, PD98059, LY294002 specification was 5 mg. All AG490, PD98059 and LY294002 powder was submerged in a bottle of water. According to the reagent specification, 0.5 ml of DMSO was added, and the inhibitors were dissolved by slightly shaking and blending and then were stored in a -20°C refrigerator.

#### *Construction of XJ-MTB-infection mouse peritoneal macrophages model*

On the ultra-clean bench at room temperature, intraperitoneally (i.p.) with 0.3 ml ( $0.3 \times 10^7$  CFU)

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**Figure 1.** The schematic picture of the macrophage grouping and inhibitor treatment. This picture shows the timing of XJ-MTB infection as well as the Mcl-1 expression-signalling pathway inhibitors AG490, PD98059, and LY294002 treatment with regard to time 0 and the 1<sup>st</sup>, 3<sup>rd</sup>, 5<sup>th</sup> and 7<sup>th</sup> days of the results discussed in the manuscript.

of the bacterial suspension to previously untreated groups of mice ( $n=10/\text{group}$ ) were infected. Uninfected control mice were administered NS on the same schedule [25]. The control and XJ-MTB-infected mice were treated i.p. with 100 µg of the signalling pathway inhibitors AG490, PD98059, LY294002 on the third day after infection (time 0). A batch of mice was taken off the neck to sacrifice every 24 hours. Then, the mouse peritoneal macrophages were harvested and cultured from each group for analysis (**Figure 1**). The method of mouse peritoneal macrophages collection and culture is according to the studies [13, 14, 26, 27].

### *Analysis apoptosis of macrophages by TUNEL assay*

The mouse peritoneal macrophages were collected in 6-well plates after infection. At room temperature, the cells were re-suspended in DMEM containing 10% FBS, and washed once in PBS before being fixed in 4% paraformaldehyde (pH 7.4) for 1 h. After an additional wash, adding a permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate in  $\text{H}_2\text{O}$ ) for 8 min on ice and washed with PBS. Finally, adding the TUNEL reaction mixture according to the manufacturer's instructions (In Situ Cell Death Detection Kit, Roche Applied Science) labelled DNA strand breaks. The number of TUNEL-positive cells was reported as a per cent of number of cells counted.

### *Determination of the CFUs*

The peritoneal macrophages from the mice were lysed 3 days after infection to release the

XJ-MTB strain. Serial 10-fold dilutions were plated on 7H9 agar, and colonies were counted after incubation for 3 weeks at 37°C to determine bacterial CFU.

### *Immunohistochemical detection of the expression of Mcl-1*

For the immunohistochemical analysis the expression of Mcl-1, mouse peritoneal macrophage smears were prepared and fixed in 4% paraformaldehyde overnight at 4°C. PBS washed the smears twice and pre-blocked for 30 min with 10% FBS (Thermo, USA) and then the samples were incubated overnight at 4°C with 1:1000 diluted polyclonal rabbit anti-mouse Mcl-1 antibody (Bioss, Beijing, China) or diluted normal rabbit serum as a control. At room temperature, smears were rinsed three times with PBS before adding 1:200 diluted biotinylated goat anti-rabbit immunoglobulin G (ZSGB BIO, Beijing, China) and then using streptavidin-peroxidase complex (ZSGB-BIO, Beijing, China) incubation with it for 30 min. Color development was achieved by applying 3'-diaminobenzidine for 3-5 min in the dark. The slides were then washed and counterstained with haematoxylin and eosin (H&E). Two pathologists performed the immunohistochemical analysis, and the sub-cellular location of Mcl-1 expression (nuclear and cytoplasmic) was recorded at 400× magnification. Positive- and negative-stained macrophages were counted, and the data were expressed as the per cent of Mcl-1-positive macrophages, as previously described. The staining pattern was classified as follows: (0) no staining or less than 5% positive cells; (1)

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**Table 1.** Primers used for real-time PCR

| Gene           | Sequence (5'-3')  |
|----------------|---|
| $\beta$ -actin | Forward: AATTCCATCATGAAGTGTGA<br>Reverse: ACTCCTGCTTGCTGATCCAC  |
| Mcl-1          | Forward: TATTTCTTTCGGTGCCTTTGTG<br>Reverse: AGTCCCGTTTCGCCTTACA |
| Bcl-2          | Forward: CGACTTCTTCAGCATCAGGA<br>Reverse: TGAGCCACAGGGAGGTTCT   |
| Bax            | Forward: TGACTGGAAGCCGAAACTC<br>Reverse: GCAAGCCATCTCCTCATCA    |
| Caspase-3      | Forward: TGACTGGAAGCCGAAACTC<br>Reverse: GCAAGCCATCTCCTCATCA    |
| Caspase-8      | Forward: GCCCTCAAGTTCCTGTGCT<br>Reverse: GATTGCCTTCTCCAACATC    |
| Caspase-1      | Forward: AATTCCATCATGAAGTGTGA<br>Reverse: CCTCCAGCAGCAACTTCATT  |
| Cytochrome-c   | Forward: CACGCTTTACCTTCGTTCT<br>Reverse: CACTCATTTCCCTGCCATTC   |

weak staining, 6-20% positive cells; (2) moderate staining, 21-50% positive cells; and (3) strong staining, more than 51% positive cells [28].

### *Transmission electron microscopy (TEM)*

Mouse peritoneal macrophages were extracted and incubated for 48 h. Later, the cells were collected, fixed in 2.5% glutaraldehyde solution and osmium tetroxide, dehydrated through a graded ethanol series to 100%, and polymerized in epoxy resin. Ultra-thin sections were collected, stained, and imaged using TEM (JEM-1230; Olympus Corporation).

### *Histopathology*

After decapitating the mice, their lungs, livers, spleens, and kidneys were immediately extracted, fixed in 10% formalin and embedded in paraffin. After adding 95% ethanol via anhydrous ethanol dehydration processing, the samples were treated with Xylene for 30 min. This step was followed by paraffin embedding, sectioning, dewaxing, 70% ethanol, 80% ethanol, 90% ethanol, 95% ethanol, and anhydrous ethanol dehydration for 5 min. Finally, samples were stained with H&E, and photographed using a CKX41 microscope (Olympus) fitted with an Olympus DP20 camera connected to a computer. Image-Pro Plus (Media Cybernetics) was used to objectively assess the level of inflam-

mation present in each image. The inflammatory areas stained a more intense purple than the non-inflammatory areas. Because of the different components, the cytoplasm showed different colours. Cells or tissue components and the lesions of the general morphology structure characteristics were shown.

### *ELISA*

Pro- and anti-inflammatory cytokines production was determined in the supernatants of MTB-infected macrophages. IL-6, TNF- $\alpha$ , IL-10, TGF- $\beta$  levels were measured by a sandwich enzyme-linked immune sorbent assay (ELISA). All assays were performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Triplicate samples were analysed using an ELISA reader and compared to a standard curve.

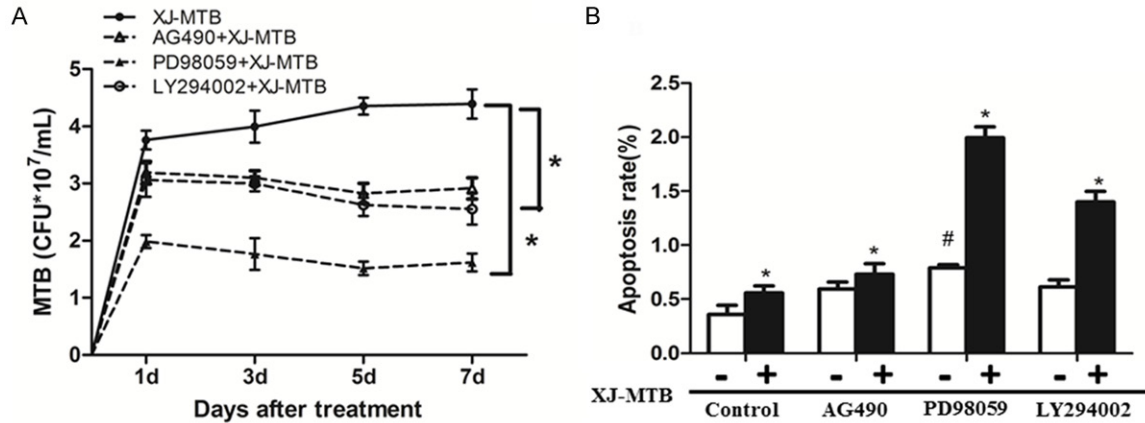
### *Quantitative real-time PCR*

Total RNA from cultured cells was extracted using TRIzol reagent (Invitrogen). Reverse transcription was performed using a SuperRT cDNA Kit (TianGen) according to the manufacturer's instructions. High quality RNA was used for reverse transcription of the first-strand cDNA synthesis by reverse transcription using a SuperRT cDNA Kit (TianGen) according to the manufacturer's instructions. Quantitative PCR primers were designed and synthesized by Sangon Biotech. The PCR reaction conditions were as follows: 95°C for 5 min, 95°C for 10 s, and 57°C for 30 s for a total of 40 cycles. Samples were amplified using the QuantiFast SYBR Green PCR Kit by following the manufacturer's instructions. The  $\beta$ -actin endogenous control was included to normalize each reaction with respect to RNA integrity, sample loading, and inter-PCR variations. The relative expression ratio was calculated from real-time PCR efficiencies and the crossing point deviation of experimental samples vs. controls. The primers sequences used in this study are listed in **Table 1**. The relative levels of Mcl-1, Bcl-2, Bax, Caspase-1, 3, 8, and Cytochrome-c mRNA were analysed using the  $2^{-\Delta\Delta Ct}$  method, and mRNA expression is shown as the fold difference compared to untreated control cells.

### *Western blotting*

Whole cell extract were washed with ice-cold PBS, and lysed in an RIPA lysis buffer (Solarbio

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**Figure 2.** Inhibitors AG490, PD98059, and LY294002 contribute to the anti-MTB effect of Mcl-1 in the peritoneal macrophages of mice. A: The peritoneal macrophages of mice classified as XJ-MTB alone, AG490+XJ-MTB, PD98059+XJ-MTB, or LY294002+XJ-MTB. Intracellular MTB levels were quantified on days 1, 3, 5 and 7 after Mcl-1 inhibitor treatment. B: TUNEL-positive cells were quantified 3 days after infection with and without Mcl-1 inhibitors. The data shown are the means  $\pm$  SDs of three independent experiments. \* $P < 0.05$  for the inhibitor-treated group compared with the untreated group. # $P < 0.05$  for the infected group compared with the uninfected groups. AG490, PD98059, and LY294002 were Mcl-1 inhibitors.

Biotechnology). The lysates were then centrifuged at  $12000 \times g$  per for 10 min at  $4^{\circ}\text{C}$ , and the supernatants were collected as whole-cell extracts. The protein concentration was measured with Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA, USA) using bovine serum albumin (BSA) as a standard. The cell extracts were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked in 4% fat free dry milk in PBS containing 0.2% Tween-20 in Tris-buffered saline-Tween-20 (TBST) and probed with a primary antibody overnight at  $4^{\circ}\text{C}$ . After washing with TBST, the membranes were probed with target-specific primary antibodies purchased from Cell Signaling Technology (CST). The blots were then developed using the enhanced chemiluminescence (ECL) reagent (Amersham Biosciences, Piscataway, NJ, USA). A Western blot analysis was performed using the following antibodies: Rabbit anti-mouse Mcl-1 and Bcl-2 (dilution: 1:10,000), Bax, Caspase-3 and Cytochrome-c (dilution 1:20,000), Caspase-8 (dilution 1:5000), and Caspase-1. Mouse anti-mouse  $\beta$ -actin (dilution 1:1000) was purchased from ZSGB-BIO followed by horseradish peroxidase-conjugated secondary antibody (Jackson Immunologicals, West Grove, PA). Blots were developed with an enhanced chemiluminescence detection system (Perkin

Elmer Bioscience, Waltham, MA) in accordance with the manufacturer's protocol.

### Statistical analysis

The data were analysed using Microsoft Office Excel 2010 and Prism (version 5.0 c, GraphPad, Inc., San Diego, CA) software. The results represent the mean and standard deviation for each experimental group. Data comparisons were performed using a one-way ANOVA followed by LSD test for multiple comparisons. These analyses were implemented using SPSS 17.0. Two-tailed unpaired Student's t-tests were used to evaluate the differences between the control and treated groups. Values of  $P < 0.05$  was considered statistically significant. All experiments repetition showed similar responses.

### Results

#### *Mcl-1 signalling pathway inhibitors limit the intracellular survival of XJ-MTB*

Studies found that cell signalling is triggered by Mcl-1 and leads to the apoptosis of macrophages infected MTB, and the major signalling pathways are JAK/STAT, PI-3K, and MAPK [15-17]. To investigate whether the signalling pathway inhibition of Mcl-1 expression was correlated with the intracellular growth of bacteria, the survival of XJ-MTB was determined in infected mouse peritoneal macrophages (Fi-

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**Table 2.** Mcl-1 expression and apoptosis rate in Mcl-1 inhibitors-treated mouse peritoneal macrophages infected with XJ-MTB on the fifth day

| Groups<br>(n=10) | Mcl-1 expression |   |    |     | Positive<br>rate (%) | Apoptosis rate<br>(%)        |
|------------------|------------------|---|----|-----|----------------------|------------------------------|
|                  | -                | + | ++ | +++ |                      |                              |
| Control          | 10               | 0 | 0  | 0   | 0                    | 0.03±0.01                    |
| XJ-MTB           | 0                | 1 | 2  | 7   | 100                  | 0.55±0.01 <sup>a</sup>       |
| XJ-MTB+AG490     | 0                | 0 | 3  | 7   | 100                  | 0.67±0.03 <sup>a,b</sup>     |
| XJ-MTB+PD98059   | 8                | 2 | 0  | 0   | 20                   | 1.83±0.03 <sup>a,b,c,e</sup> |
| XJ-MTB+LY294002  | 0                | 1 | 2  | 6   | 90                   | 1.27±0.02 <sup>a,b,d</sup>   |

Note: <sup>a</sup>*P*<0.05 vs. control group; <sup>b</sup>*P*<0.05 vs. XJ-MTB infection group; <sup>c</sup>*P*<0.05 vs. AG490+XJ-MTB; <sup>d</sup>*P*<0.05 vs. PD98059+XJ-MTB; <sup>e</sup>*P*<0.05 vs. LY294002+XJ-MTB.

**Figure 2**) that were untreated or treated with the inhibitors AG490, PD98059, and LY294002. Significant decreases were observed in the number of colony-forming units recovered from PD98059-treated cells compared with the number from untreated XJ-MTB-infected cells (**Figure 2**; *P*<0.05). The survival rate of XJ-MTB was decreased by approximately 60% in the PD98059-treated mouse peritoneal macrophages.

### *Mcl-1 signalling pathway inhibitors increased mouse peritoneal macrophage apoptosis*

MAPK activity is central to the activation of the diverse immunological mechanisms in macrophages [29]. Given that both MAPK signalling pathway reduced the intracellular growth of bacteria in mouse peritoneal macrophages, we hypothesized that the MAPK pathway might be in a dominant position in induced macrophage apoptosis. To investigate this possibility, the apoptosis rate of the JAK/STAT, MAPK, and PI-3K signalling pathways were examined via terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL). After the JAK/STAT, MAPK, and PI-3K signalling pathways inhibitors alone inhibited Mcl-1 expression in mouse peritoneal macrophages, only the apoptosis rate of the PD98059-treated group showed a slight increase compared with that of the untreated group (**Figure 2**; *P*<0.05). However, the apoptosis rates significantly increased in AG490, PD98059, and LY294002-treated peritoneal macrophages from XJ-MTB-infected mice compared with that in the XJ-MTB-infected group (**Figure 2**; *P*<0.05). Furthermore, the apoptosis-inducing effects in the PD98059-treated group were significantly stronger than

those in the AG490 and LY294002-treated groups (approaching 2.7 times and 1.4 times, respectively), and these differences were significant (**Table 2**; *P*<0.05).

### *PD98059 treatment suppressed Mcl-1 expression in host macrophages*

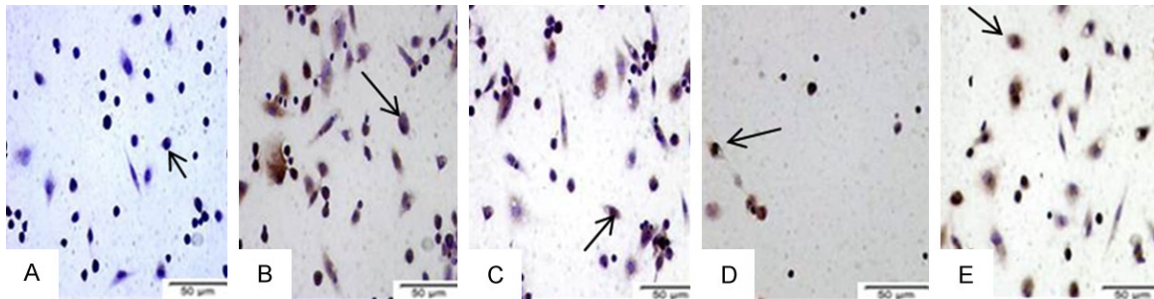
Based on our previous results showing that Mcl-1 protein expression was more significantly decreased after the MAPK signalling pathway inhibitor PD98059 than PI-3K and JAK/STAT pathway inhibition [14], we hypothesized that the MAPK regulatory path-

way is the most important for Mcl-1 expression in MTB infection. Immunostaining of the Mcl-1 protein in mouse peritoneal macrophages revealed that it was predominantly localized in the cytoplasm and nucleus (**Figure 3**). Our data showed strong staining for Mcl-1 in the peritoneal macrophages of mice infected with XJ-MTB (**Figure 3B**). In addition, diffuse staining for Mcl-1 was obvious in uninfected host cells (**Figure 3A**). Staining for Mcl-1 in mouse peritoneal macrophages treated with the inhibitors AG490, PD98059, and LY294002 was weakened. These results corroborate our previous findings: The expression of Mcl-1 is significantly decreased in the peritoneal macrophages of PD98059-treated mice infected with XJ-MTB compared with that in the XJ-MTB infection group with only 20% positive cells (**Figure 3**; **Table 2**; *P*<0.05).

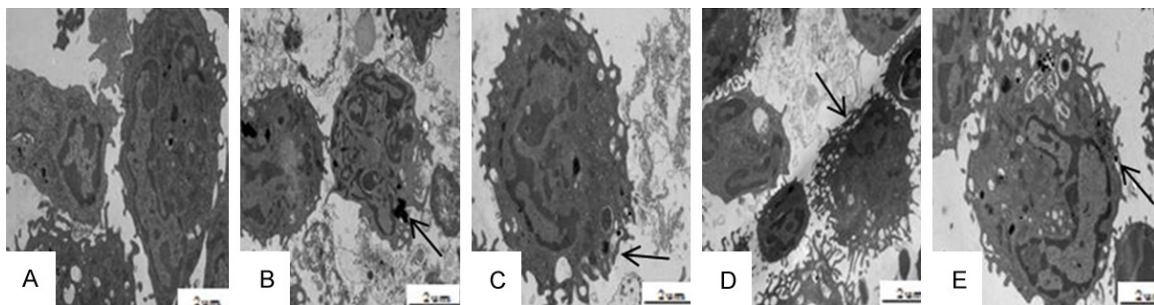
### *The MAPK pathway is almost more important than the JAK/STAT and PI-3K pathways in mice infected XJ-MTB*

The induction of apoptosis in host macrophages following intracellular infection with XJ-MTB was also confirmed via transmission electron microscopy (TEM). Our data showed that both autophagic and phagocytic vacuoles were induced in the peritoneal macrophages of mice infected with XJ-MTB (**Figure 4B**). Host macrophages destroyed the structural integrity of the XJ-MTB cell wall to achieve pathogen clearance. Treatment with the inhibitors AG490, PD98059, and LY294002 induced nuclear pyknosis and apoptosis in the mouse peritoneal macrophages to kill the intracellular infection with XJ-MTB, especially the MAPK signalling pathway inhibitor PD98059 (**Figure 4C-E**).

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**Figure 3.** Typical Mcl-1 immunohistochemistry results for the peritoneal macrophages of mice ( $\times 400$ ). A: Control cells: Less than 5% of macrophages showed detectable cytoplasmic or nuclear expression of Mcl-1 protein. B: Control cells infected with XJ-MTB: Strong staining for Mcl-1 was detected. C: XJ-MTB-infected host cells treated with AG490. D: XJ-MTB-infected host cells treated with PD98059. E: XJ-MTB-infected host cells treated with LY294002. After PD98059 treatment, weak staining was detected for Mcl-1. Arrows indicate the observed host cells.



**Figure 4.** Inhibitor PD98059 reduced XJ-MTB infection in the peritoneal macrophages of mice. TEM was used to observe the morphology of peritoneal macrophages obtained from XJ-MTB-infected, uninfected, AG490-treated, PD98059-treated, LY294002-treated and untreated mice. Images of the peritoneal macrophages of mice in the (A) control cells, (B) control cells infected with XJ-MTB, (C) XJ-MTB-infected cells treated with AG490, (D) XJ-MTB-infected cells treated with PD98059, and (E) XJ-MTB-infected cells treated with LY294002 (magnification,  $\times 40,000$ ). Arrows indicate the observed host cells.

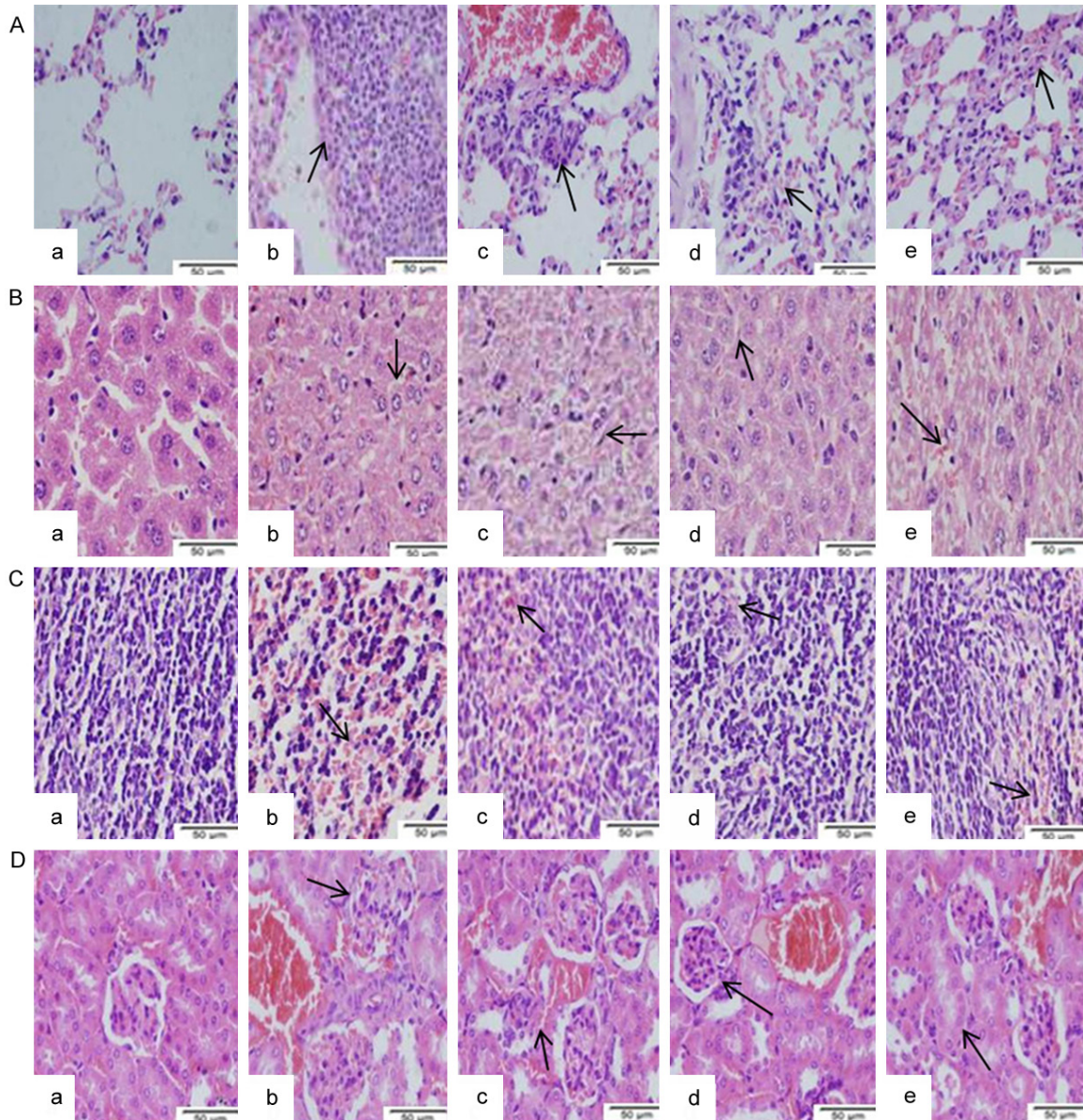
### *Mcl-1 inhibitor treatment relieved the pathological damage of mouse organs*

To verify whether the inhibitors of Mcl-1 expression play a role in MTB infection and the apoptosis of mouse macrophages, we observed the pathological damage of the lung, liver, spleen, and kidney before and after the inhibitor treatment in mice. After XJ-MTB infection in the lung, multifocal epithelioid cells were observed in the lung tissue. Numerous lymphocytic infiltrations of nodular lesions, alveolar capillaries expansion and congestion, interlobular vein expansion, along with liver cell oedema, cavitation and degeneration around the central vein cavity, and significant central vein blood clotting were observed. Moreover, liver antrum narrowing, bleeding in a partial area, and interstitial lymphocytic infiltrates were found. A greater quantity of mononuclear macrophages was found in splenic sinus congestion and expan-

sion. In addition, significant glomerular and renal interstitial blood clotting, focal haemorrhage, and tubular epithelial cells oedema were observed (**Figure 5A-D**).

The AG490-treated group showed almost no relief from the XJ-MTB infection-caused pathological damage (**Figure 5A-D**). After LY294002 treatment, lung interlobular vein expansion was reduced, liver oedema was relieved, and spleen leucocyte ooze decreased. However, PD98059 treatment significantly relieved pathological organ damage; alveolar capillaries mildly expanded and congestion declined, the number of epithelioid cells reduced, and lymphocyte seepage decreased. The degrees of liver cell oedema, congestion, and haemorrhage were reduced. Spleen sinus congestion degree reduced, and leucocyte seepage decreased. Moreover, kidney congestion degree decreased,

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**Figure 5.** Histological changes in XJ-MTB-infected mice treated with the inhibitors AG490, PD98059, LY294002. A-D: Display the H&E staining of normal and inhibitor-treated XJ-MTB-infected lung, liver, spleen, and kidney tissues in mice. A: Normal lung tissue and inhibitor-treated XJ-MTB-infected lung tissue. B: Normal liver tissue and inhibitor-treated XJ-MTB-infected liver tissue. C: Normal spleen tissue and inhibitor-treated XJ-MTB-infected spleen tissue. D: Normal kidney tissue and inhibitor-treated XJ-MTB-infected kidney tissue. a. Control group: normal mouse tissue. b. XJ-MTB-infected mouse tissue. c. AG490-treated XJ-MTB-infected mouse tissue. d. PD98059-treated XJ-MTB-infected mouse tissue. e. LY294002-treated XJ-MTB-infected mouse tissue. Arrows indicate the pathological changes of lung tissue.

and epithelial cell oedema was alleviated (**Figure 5A-D**).

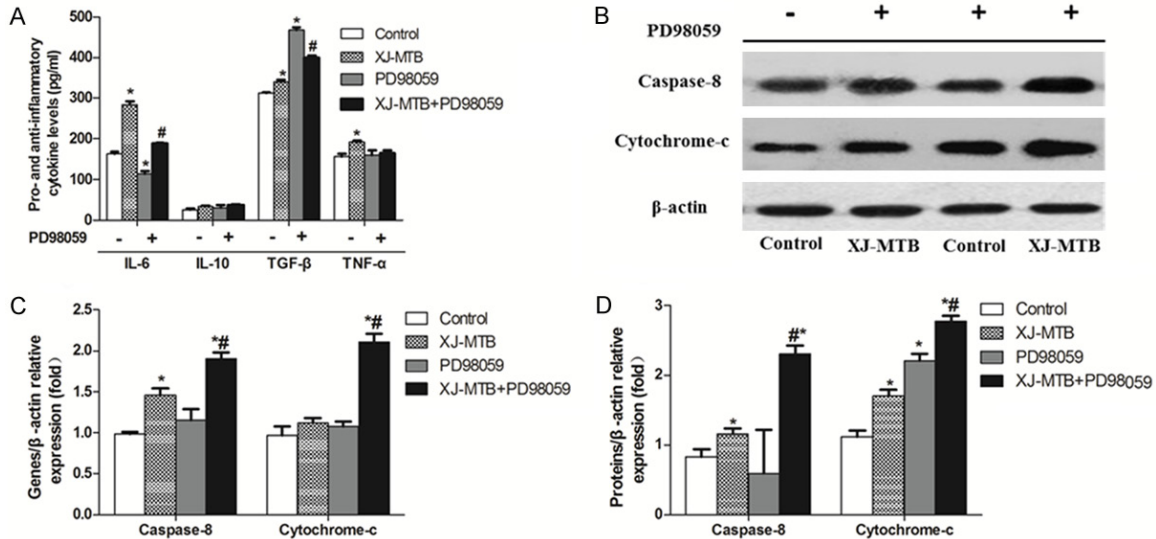
*Inhibitor PD98059 treatment promotes apoptosis and is conducive to the elimination of inflammation*

As shown in **Figure 5**, after inhibitor PD98059 treatment significantly reduced the pathologi-

cal damage of mice organs, so we speculated that by PD98059 inhibit the expression of Mcl-1 promoted the mycobacterium tuberculosis infection of host macrophage apoptosis, thus reduce the inflammatory response. Therefore, pro- and anti-inflammatory cytokines IL-6, TNF- $\alpha$ , IL-10 and TGF- $\beta$  were detected by ELISA, apoptotic protein Caspase-8 and Cyto-



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**Figure 6.** The expression of inflammatory cytokines and Caspase-8, cytochrome-c mRNA and protein after PD98059-treated. (A) Secretion of IL-10, IL-6, TNF- $\alpha$  and TGF- $\beta$ . Mouse peritoneal macrophages were treated with XJ-MTB in the fifth day. Supernatant was collected for IL-10, IL-6, TNF- $\alpha$  and TGF- $\beta$  production by ELISA. (B-D) Western blot detected the expression of Caspase-8 and cytochrome-c mRNA and protein. (C) RT-PCR detected the expression of Caspase-8 and Cytochrome-c mRNA. Total RNA was obtained from Raw264.7 cells and mouse peritoneal macrophages. For all groups (BCG-infected, H37Rv-infected, uninfected, PD98059-treated, untreated), quantitative PCR was performed on the cDNA. Each group of protein lysates were separated by using SDS-PAGE (12% and 15%). Results in (A-D) are means  $\pm$  SD from individual experiments each with 3 replicates that are representative of at least 2 independent experiments.

chrome-c levels were detected by Real-time PCR and western blot (Figure 6). Pro-inflammatory cytokines IL-6 and TNF- $\alpha$  were significantly increased after XJ-MTB infection compared with the control group, while IL-6 was significantly decreased after inhibitor PD98059 treated in mouse peritoneal macrophages, especially PD98059-treated XJ-MTB infection group (Figure 6A;  $P < 0.05$ ). Anti-inflammatory cytokines IL-10 and TGF- $\beta$  levels were significantly increased after inhibitor PD98059 treated in mouse peritoneal macrophages (Figure 6A;  $P < 0.05$ ).

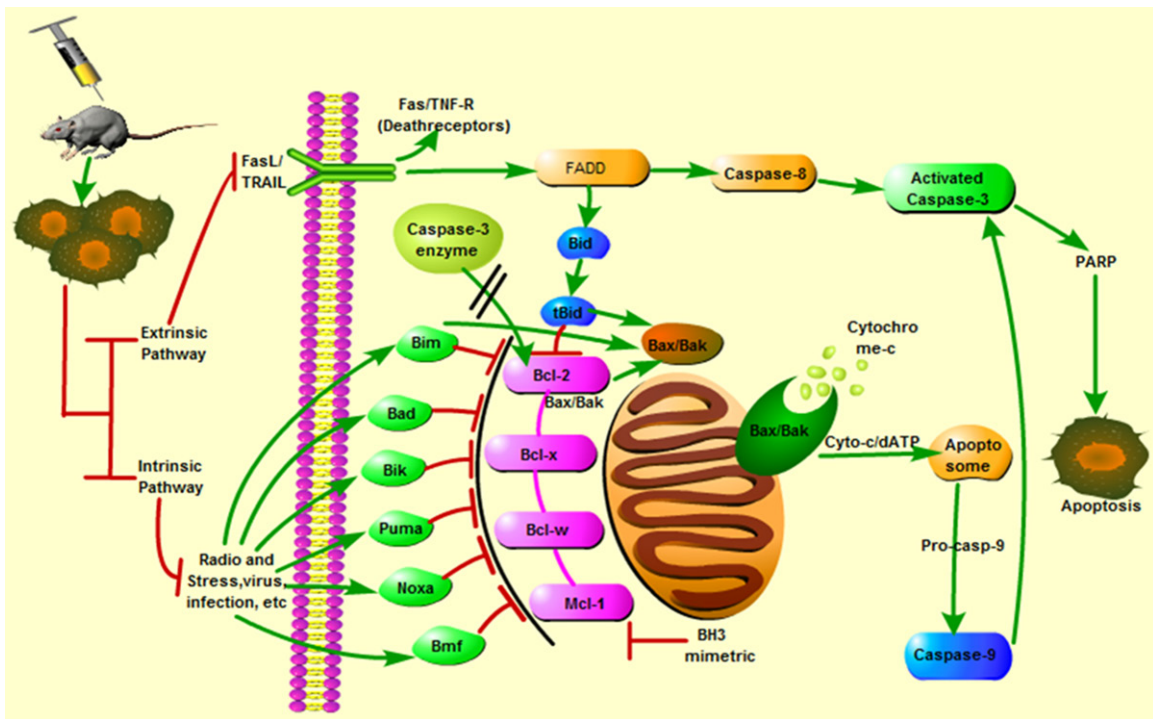
Considering the important role of Caspase-8 and Cytochrome-c in the extrinsic apoptotic pathway and the intrinsic apoptotic pathway [28, 30], we detected Caspase-8 and Cytochrome-c mRNA and protein levels in inhibitor PD98059-treated XJ-MTB-infected mouse peritoneal macrophages. After XJ-MTB infection, Caspase-8 mRNA was increased compared with the control group, and after PD98059 treatment, Caspase-8 and Cytochrome-c mRNA were increased compared with XJ-MTB infection groups in mouse peritoneal macrophages

(Figure 6C;  $P < 0.05$ ). Likewise, Caspase-8 and Cytochrome-c protein levels were significantly increased before and after inhibitor PD98059-treated, and Cytochrome-c levels was higher than Caspase-8 (Figure 6B and 6D;  $P < 0.05$ ).

### *The mechanism hypothesis about Mcl-1 in MTB infected host macrophages apoptosis*

The regulatory mechanism of inhibiting Mcl-1 promotes host macrophage apoptosis is inseparable from the intrinsic and extrinsic apoptotic pathways [31, 32]. The extrinsic apoptotic pathway is determined by the expression of Caspase-8, death receptor Fas combined with the corresponding ligand binding, and Caspase-8 activated by the FADD bridging protein; finally, apoptosis execution enzyme Caspase-3 induced apoptosis [30]. The intrinsic apoptotic pathway that activates the endogenous protease enzyme system (Caspases) and the Bcl-2 family regulation are inseparable [31-33, 11]. With reference to many studies, we have drawn the mechanism hypothesis about MTB infection in host macrophages apoptosis (Figure 7).

## The role of Mcl-1 in XJ-MTB infection



**Figure 7.** Mcl-1 regulatory mechanism hypothesized in MTB infection. After MTB infected the host macrophages, the related apoptosis regulatory mechanism refers to the extrinsic apoptotic pathway and the intrinsic apoptotic pathway. This picture shows the major signalling transduction relationship in the two apoptotic molecular pathways.

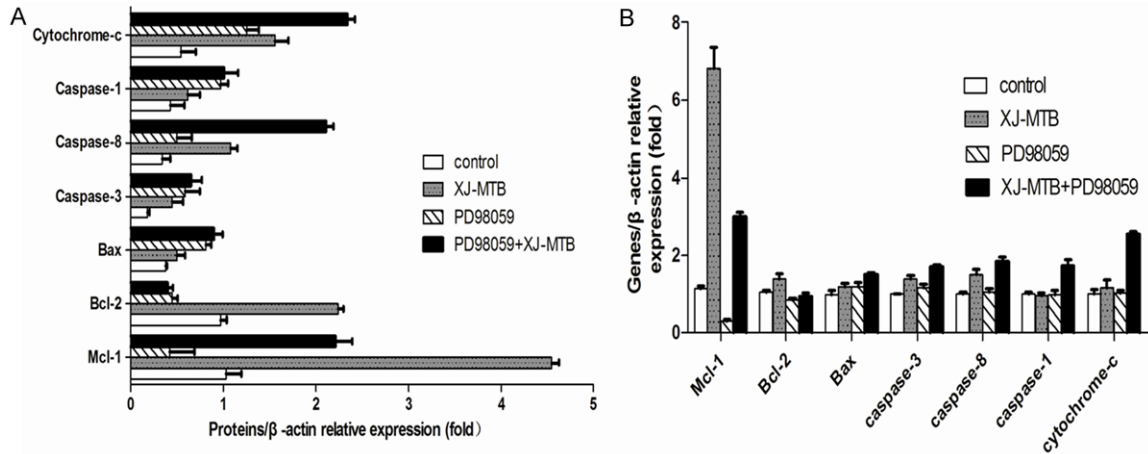
The Caspase family has a type of aspartic acid residue that is specific to their cysteine protease activity and has an important enzyme modification effect on Bcl-2 family proteins (e.g., Mcl-1, Bax, and Bcl-2). This effect can be detected by the different levels of Caspase that are increased during the process of infected-cell apoptosis; the expression changes determine the developmental direction of cell apoptosis [34]. Although Caspase-3 is in the core position of apoptosis after the influence of apoptosis signals stimulation and damage in cells (e.g., via radio waves, stress, infection, and virus), this enzyme is spliced and activated by a different protease. Activated Caspase-3 can activate different substrates after cutting and amplifying the protease cutting cascade, which activates nuclease, prompts the DNA between nuclear corpuscles via pyrolysis, causes chromatin concentration and cell shrinkage, and eventually prompts the formation of the apoptosome, leading to cell apoptosis [35].

When cell apoptosis occurs, Bcl-2 is digested by the Caspase-3 enzyme. After transfection to BHK, BHK can also be translocated to the mitochondria to promote the release of Cyto-

chrome-c in mitochondria and accelerate cell apoptosis [36]. Although Bcl-2 is the first recognized human longevity gene, at least three levels of mitochondrial membrane function exist to inhibit apoptosis. Within the Bcl-2 and Bax interaction in the target mitochondria organelles, the role of Bcl-2 depends on not only the level of gene expression but also the Bcl-2-Bax ratio [37]. Normally, Bax and Bcl-2 form the heterodimer Bax-Bcl-2, and the two genes change their integration into the membrane required for conformation and cannot form the membrane channel to promote cell survival, thereby inhibiting cell apoptosis. When Bax increases, however, it forms the homodimer Bax-Bax and is given priority to promote cell apoptosis. Under the apoptosis stimulus signal, the Bax homodimers shift from the cytoplasm to the mitochondrial membrane and cause changes in mitochondrial permeability to cause the mitochondria to release Cytochrome-c into the cytoplasm, thereby activating the Caspase cascade and causing apoptosis [38].

Mitochondria are not only the control centre of the cellular respiration chain and oxidative phosphorylation but also control cell apoptosis

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**Figure 8.** Apoptosis-related gene and protein expression comparison. Real-time PCR and Western blot analyses detected the expression of the apoptosis-related genes and proteins Mcl-1, Bcl-2, Bax, Caspase-1, 3, 8, and Cytochrome-c after the inhibitor PD98059 was used to treat the peritoneal macrophages of mice. A: Real-time PCR was used to detect the relative expression of Mcl-1, Bcl-2, Bax, Caspase-1, 3, 8, and Cytochrome-c mRNA. B: Western blot was used to detect the relative expression levels of Bcl-2, Bax, Caspase-1, 3, 8, and Cytochrome-c protein. The data shown are the means  $\pm$  SDs of three independent experiments.

regulation. The Bcl-2 family contains BH3 structural domain members that can combine with Bax loosely in the mitochondrial outer membrane, leading to the combination of the Bax oligomer and insert into the mitochondrial membrane, changing the mitochondrial outer membrane permeability and leading to the loss of transmembrane potential, thereby freeing Cytochrome-c [39]. The main step of the intrinsic apoptotic pathways is the release of Cytochrome-c. Researchers think that when mitochondrial respiratory frequency increases, the mitochondrial permeability transition pore PTP opens and the Cytochrome-c that is released by the mitochondrial membrane releases into the cytoplasm in the presence of dATP/ATP conditions, which can combine with factors that are associated with apoptosis, activating Caspase-9. Therefore, activated Caspase-3 leads to apoptosis [40]. However, the study found that the activation of Caspase-8 and Caspase-3 can degrade Mcl-1 and release BIM, which is combined with Mcl-1, leading to apoptosis. Mcl-1 expression can dramatically decrease after apoptosis, and this decrease might enhance the mitochondria's outer membrane permeability to Cytochrome-c to promote apoptosis [40]. Therefore, exploring the regulatory mechanism of Mcl-1 must analysis comprehensively the changes of apoptotic proteins in apoptotic pathways before and after inhibitor PD98059-treated.

### *The regulatory mechanism of PD98059 in host macrophage apoptosis infected with XJ-MTB*

The regulation of programmed cell death occurs either via the intrinsic pathway involving the mitochondrial release of Cytochrome-c or the extrinsic pathway involving the stimulation of the death receptors expressed on the cell surface and the activation of Caspase-8, both eventually lead to the activation of effector caspases (Caspase-3) to execute cell death [31, 32]. Mycobacteria interfere with host apoptosis by modifying Bcl-2 protein expression, and virulent MTB represses apoptosis via the up-regulation of Bcl-2 [33] and Mcl-1 [11] as well as the deactivation of Bad [42] and Bax [43]. Thus, apoptosis-related genes and the proteins Bcl-2, Bax, Caspase-1, 3, 8, and Cytochrome-c were detected via real-time PCR and Western blot analyses.

After XJ-MTB infected the peritoneal macrophages of mice, all detected protein levels increased, whereas Mcl-1, Cytochrome-c and Bcl-2 protein levels were higher than those of other proteins, and the Mcl-1 protein level was highest (**Figure 8B**;  $P < 0.05$ ). After treatment with the inhibitor PD98059, the peritoneal macrophages of mice infected with XJ-MTB showed Mcl-1 and Bcl-2 protein levels that were significantly decreased, but Caspase-8 protein levels that were significantly increased. The

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Cytochrome-c protein level remained high compared with that in the untreated XJ-MTB infection group, and higher than Caspase-8 protein levels (**Figure 8B**;  $P < 0.05$ ). However, mRNA expression of the apoptosis-related genes showed almost no significant changes (**Figure 8A**;  $P > 0.05$ ).

### Discussion

Since the advent of effective anti-TB drugs and the associated improvements in standards of living and health status, the TB epidemic has fallen sharply. Recently, however, TB rates have risen because of the incidence of drug-resistant MTB; furthermore, the BCG vaccine provides poor immune protection for TB. The abuse of immunosuppressants coupled with the prevalence of HIV infection, the disease spectrum change, the speed of population flows, and so on, they have caused TB resurgence [1]. Importantly, Xinjiang is a high incidence region of TB. Therefore, determining the existing problems and taking corresponding measures is necessary and important to effectively control the TB epidemic in the Xinjiang region.

Studies have found that host cell apoptosis plays an important role in the natural defence of anti-infection immunity [44, 45], and the apoptosis of macrophages is important to the fate of MTB infection [46]. Thus, the normal procedure of macrophage apoptosis is the key to controlling sustained and latent TB infection. Our experimental group previous study found that the anti-apoptotic protein Mcl-1 plays an important role in the interaction between MTB and host macrophages. Therefore, the current study was explored the regulatory role of Mcl-1 signals pathways in XJ-MTB-infected mouse peritoneal macrophages apoptosis. TUNEL results showed that all Mcl-1 signals pathways inhibitors-treated macrophage apoptosis rates were increased compared with that the untreated group, whereas inhibitor PD98059 induced more macrophages apoptosis (**Figure 2**). These results indicate that inhibiting the Mcl-1 signalling pathway by inhibitors AG490, PD98059, and LY294002 could increase XJ-MTB-infected host macrophages apoptosis. What's more, Colony-forming unit (CFU) results found that the survival rate of XJ-MTB in the peritoneal macrophages of PD98059-treated mice significantly

decreased (**Figure 2**). These findings indicate that PD98059 inhibits the expression of Mcl-1 increase XJ-MTB infected host macrophage apoptosis and it help to kill latent MTB. The experimental results revealed that the MAPK signalling pathway plays an important role in XJ-MTB-infected host macrophage apoptosis.

However, regulating the Mcl-1 expression involved in these signalling pathways is extremely complex *in vivo*. The signalling pathways might work alone, or they might mutually coordinate. Previous studies [24, 47-49] have shown that the main signalling pathway involved in regulating Mcl-1 expression is different in disparate cells and tissues. The cell immunochemistry results showed that the expression level of Mcl-1 was lowest when treated with PD98059 in XJ-MTB-infected mouse peritoneal macrophages (**Figure 3**), which confirmed that the MAPK signalling pathway is the major signalling pathway involved in regulating the expression of Mcl-1 in XJ-MTB infected host macrophages. Similarly, the TEM results also verified this conclusion (**Figure 4**). The reason for this result might be associated with the several subtypes of MAPK structure. The MAPK family is an important hub of cell signal transduction; the MAPK subtypes and family can be adjusted by manipulating a variety of molecular mechanisms involved in the body's immune response. In addition, a variety of biological factors (such as growth factors, lymphatic factors, promoting factors of the tumour, emergency factors) can activate the MAPK pathway through the membrane receptor, and part of the activated MAPK can be applied directly to the cell membrane and target molecules in the nuclear membrane (the rest migrate within the nucleus) to participate in cell proliferation, differentiation, metabolism, apoptosis, and so on. Therefore, the specific inhibitor PD98059 inhibits the MAPK signal transduction pathway and might inhibit other signalling pathways involved in regulating Mcl-1 expression via the indirect regulation of MAPK.

As mentioned above, studies suggested that the RAS-RAF-MEK-ERK signalling pathway leads to the activation of the PI-3K/AKT pathway [50]. After MTB infects macrophages, JAK2/STAT1- $\alpha$  phosphorylation most likely occurred via the activation of tyrosine protein kinase, which led to JAK2/STAT1- $\alpha$  phosphory-

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lation, activated STAT1- $\alpha$ -induced cells and generated numerous TNF- $\alpha$  molecules [51], leading to an increase in Caspase enzyme system activity and the start of apoptosis. Therefore, the decomposition of the Caspase enzyme system into Mcl-1 might be the main reason for the down-regulation of Mcl-1. The blocking of the JAK/STAT, MAPK and PI-3K signalling pathways via AG490, PD98059 and LY294002 treatment restrained the role of TNF- $\alpha$ , thus, the Caspase enzyme activity was reduced, and the Mcl-1 expression level rose. Although the inhibitory effect of TNF- $\alpha$  caused Mcl-1 to increase, but adversely affected the decrease of Mcl-1 from the signalling pathway inhibitors, so the expression of Mcl-1 in the AG490 and LY294002 treatment groups showed almost no significant changes. Nevertheless, p38 MAPK and two branches of ERK regulated TNF- $\alpha$  in the MAPK signalling pathways [52]. So there's no question of the effect of the PD98059 treatment group was the most obvious to interfere with the expression of Mcl-1 in our experiment. Moreover, the induced macrophage apoptosis rate was highest. Furthermore, p38 MAPK pathway activation generated numerous TNF- $\alpha$  molecule, it also leading to an increase in Caspase enzyme activity, thereby down-regulating the expression of Mcl-1. These results confirm that inhibitor PD9809 play the key role in inhibiting Mcl-1 expression in MTB infection.

In addition, improvement in the pathological damage of mouse organ tissue is the most important observational index that specifically reflects the effect of inhibitors in the current study. In this study, MAPK signalling pathway inhibitor PD98059 treatment significantly relieved the pathological damage to the lung, liver, spleen, and kidney in mice compared with damage in the controls, especially PD98059-treated XJ-MTB infection group of mice (**Figure 5**). What's more, the application of inhibitor PD98059 was also increase the levels of anti-inflammatory cytokine IL-10 and TGF- $\beta$  in XJ-MTB mouse peritoneal macrophages (**Figure 6**). These results indicate that inhibitor PD98059 not only relief the pathological damage of mice tissues, but also it is good for the elimination of inflammation. Therefore, introducing the Mcl-1 signalling pathway inhibitor PD98059 inhibiting the expression of Mcl-1 to control TB latent infection will be a potential strategy. Of

course, considering the important role of Mcl-1 in MTB latent infection, so the regulatory mechanism of Mcl-1 in MTB-infected host macrophages apoptosis is necessary to explore.

The regulation of cell apoptosis is inseparable from the function of the intrinsic pathway and the extrinsic pathway [31, 32]. Thus we analysed the key protein of the two apoptotic pathways Cytochrome-c and Caspase-8 expression. Real-time PCR and western blot results showed that inhibitor PD98059 treatment was significantly increased the expression of Cytochrome-c and Caspase-8 protein, but Cytochrome protein levels was higher than Caspase-8 (**Figure 6**). The results indicate that the regulatory mechanism of PD98059 in XJ-MTB-infected mouse peritoneal macrophages is closely related to the extrinsic apoptotic pathway and the intrinsic apoptotic pathway. However, there are many proteins that regulate apoptosis in MTB infection (**Figure 7**). Therefore, it is necessary to comprehensively analysis the changes of related proteins in apoptosis pathways to explore its regulatory mechanism.

The study found that inhibiting the expression of Mcl-1 in H37Rv-infected host macrophages might directly or indirectly control the expression of Bcl-2 and Bax, thereby affecting host macrophage apoptosis [13]. Besides, Caspases family also plays an important role in cell apoptosis. For this reason, we detected the expression of apoptosis-related genes and protein Mcl-1, Cytochrome-c, Caspase-1, 3, 8, Bcl-2 and Bax to explore the regulatory mechanism of Mcl-1. The Western blot results showed that Mcl-1, Cytochrome-c, and Bcl-2 protein levels significantly increased after XJ-MTB infection alone compared with the levels in the control group. Although inhibiting Mcl-1 expression via the inhibitor PD98059 significantly increased Cytochrome-c and Caspase-8 expression compared with that the untreated group, and the expression level of Cytochrome-c was significantly higher than the Caspase-8 protein level (**Figure 8**). Mcl-1 was significantly reduced, and the Bax protein level slightly increased. These data suggest that the intrinsic apoptotic pathway plays a predominant role in XJ-MTB infection.

In conclusion, the MAPK signalling pathway inhibitor PD98059 down-regulated Mcl-1 ex-

pression and effectively increased macrophage apoptosis in mice infected with XJ-MTB. Furthermore, it also relief pathological organ damage and promote the elimination of inflammation. The intrinsic apoptotic pathway plays a predominant role in the regulatory role. The introduction of Mcl-1 signalling pathway inhibitor PD98059 to control and prevent the latent and persistent infection of XJ-MTB provides a new direction and opportunity.

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All studies were conducted following the principles stipulated by the Institutional Animal Ethics Committee (IAEC).

### Disclosure of conflict of interest

None.

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