

WITHDRAWN: GPR84 inhibitor ameliorated lung inflammation and modulated NF- κ B through MAPK signalling pathway

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EDITORIAL NOTE:

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Abstract

We set our experiments to investigate the role of GPR84 in LPS-induced acute lung injury. The expression of GPR84 was detected through RT-qPCR and Western blotting (WB) after LPS treatment in ALI mouse and cell model, respectively. Following the treatment of GPR 84 inhibitor, the lung injury was evaluated through HE staining while the MAPK signalling components were analyzed through WB, as well as NF- κ B signalling components. These components were further analyzed by the addition of anisomycin or TPA. The analysis of apoptosis was performed through TUNEL staining. We showed that LPS stimulated the expression of GPR84 expression. GPR 84 inhibitor facilitated the activation of MAPK signalling while inhibiting MAPK signalling, these effects of which were further rescued by anisomycin or TPA treatment. In conclusion, our findings support that GPR84 inhibitor improved lung injury caused by LPS and revealed a role of GPR87 modulating NF- κ B through MAPK signalling pathway. Altogether, GPR84 could be as a prospective target for the treatment of ALI.

Introduction

Inflammatory response plays an important role in the pathophysiological process of acute lung injury. In the early stage of ALI, local neutrophils and macrophages infiltrated, releasing various cytokines such as IL-6, IL-1 β and TNF- α , which were expressed in the systemic and local tissues. If the inflammation was not controlled, leukocytes would be further activated, causing systemic inflammatory response syndrome[1]. As a kind of membrane protein receptor, GPR84 contains seven transmembrane alpha helix structures, and each transmembrane region contains 25–35 amino acid residues. GPR84 is generally expressed at a low level under normal conditions, the high expression of which can be induced by the presence of certain inflammatory stimuli. Studies found that expression levels of GPR84 were induced by pro-inflammatory signals, including TNF- α , IL-1 β and IL-33[2, 3]. Gpr84 has increased expression in lung damaged tissues and can participate in the regulation of the low expression state of CD11b to the high expression state of CD11b in alveolar macrophages [4].

The marked upregulation of IL-33 was found in lung tissue induced by sepsis, which regulated inflammation and accelerated lung injury, which could relate to the regulation of AMPK signally[5–7]. In ALI, MAPK pathway is activated by pro-inflammatory cytokines such as LPS, which plays vital roles in regulating inflammatory response, promoting the apoptosis of cells in lung tissue and leading to endothelial dysfunction in lung tissue and damaging to cellular microtubule structures[8].

A study found that Inhibiting GPR84 markedly lead to decreases in macrophage accumulation and inflammation[9]. Additionally, the knockdown of GPR84 induced the increase of apoptosis[10]. A study also indicated a role of GPR84 in regulating inflammation and apoptosis. Mechanistically, the function of GPR84 may be related to the modulation for NF- κ B and MAPK signaling pathways[11]. The present study intended to decipher the role of GPR84 in lung injury induced by LPS in vivo and vitro and illustrate it potential action mechanism.

Method

Animal experiment

C57BL/6 mice of SPF grade were purchased from Guangdong Medical Experimental Center (Guangdong, China). Mice were randomly divided into control group and LPS group. Mice in LPS group were given 5 mg.kg⁻¹ LPS (LPS was dissolved into phosphate-buffered saline (PBS)) by intratracheal instillation, respectively.

Cells culture

BEAS-2B cells were obtained from ATCC (USA) and cultured in DMEM medium with 10% FBS at 37 °C with 5% CO₂. When the cells grew to the logarithmic stage, the cells were digested with 0.25% trypsin digestion solution containing EDTA. The cells were resuspended with medium and used for further experiments.

Western blot

Lung tissue or cell proteins were subjected to SDS-PAGE gel electrophoresis, which then were transferred to the PVDF membrane. 5% skim milk was used to block proteins at room temperature for 1h. Primary antibody (Abcam, England) was added for incubation overnight at 4°C. After washing with PBS, the secondary antibody was added for incubation at room temperature for 1 h. ECL chemiluminescence chromogenic solution was used to develop the imaging. Image J was used to measure the gray value of each strip.

RT-qPCR

The total RNA of lung tissue or cells was extracted using Trizol reagent, which was used as plates of cDNA synthesis using TOYOBO reverse transcription kit. The PCR reaction system was prepared by using cDNA as template. The relative mRNA levels were calculated using $2^{-\Delta\Delta C_t}$ method.

HE staining

The lung tissues of the mice were placed in 5% paraformaldehyde solution, embedded in paraffin, sliced and stained with hematoxylin eosin (HE). The histopathological changes of the lungs were observed under light microscope. Three fields were randomly selected for each slice.

Lung dry/wet ratio

The lung tissue was completely removed and the remaining bronchi and connective tissue were carefully removed with surgical scissors. After washing the lung tissue in ultrapure water, the water was wiped out with absorbent paper. The tissue was placed in a 60 °C incubator for 72h, then the dry weight was weighed. The wet/dry mass (W/D) ratio was calculated.

ELISA

The expressions of TNF- α , IL-1 β and IL-6 in the supernatant were detected according to the instructions of ELISA kit. Reagents should be prepared according to the instructions while avoiding foaming as much as possible. The supernatant samples of each bronchoalveolar lavage fluid to be measured were added to the sample holes, respectively. The samples were taken for three repetitions, gently shaken and mixed, covered with a plate and incubated at 37°C for 2h. The absorbance of each hole to be measured was read successively at the wavelength of 450nm with a microplate reader.

Myeloperoxidase (MPO) kit

The blood and exudate of the lung tissue of mice were sucked dry with filter paper and rinsed in cold normal saline. After being dried with filter paper, the lung tissue of appropriate weight was weighed, which then was prepared into 5% lung tissue homogenate at the ratio of 1:19 of the lung tissue and reagent solution. The OD value was detected at 460nm with a microplate reader.

TUNEL assay

The apoptosis levels were detected using TUNEL kit according to manufacturer's guidance (Roche). The 3'-OH terminus of broken DNA was labeled with deoxyribonucleotide and fluorescein. We randomly selected 5 non-overlapping fields to observe cell apoptosis levels under a fluorescence microscope. The ratio of positive cells levels in each field and all cells levels in the field was calculated as the apoptosis levels of cells.

Statistical analysis

GraphPad Prism 8.0 software was used for statistical analysis. All experimental data were represented by mean \pm SD and the comparison among groups was performed by one-way ANOVA, followed by turkey's test. $P < 0.05$ indicated that the difference was statistically significant.

Result

The upregulation of GPR84 expression induced by IL-33

We firstly evaluated the expression of GPR84 through detecting its transcription and translation in lung tissue in LPS-induced mice and BEAS-2B cells (Fig. 1A-B). The results showed that GPR84 was significantly upregulated through LPS treatment. Furthermore, the upregulation of GPR84 was further induced by the co-treatment of LPS and IL-33 both lung tissue (Fig. 1C-D) and pulmonary epithelial cells (Fig. 1E-F).

GPR84 inhibitor ameliorated lung injury

In this experiment, after LPS-induced ALI in mice for 12h, the lung lobules of mice in the control group were intact, with no abnormal alveolar walls, no infiltration of inflammatory cells and no alveolar exudation (Fig. 2A). The lung tissues of mice in LPS group were infiltrated by a large number of

inflammatory cells and the alveolar septa were thickened. In addition, the alveolar interstitium is marked with hyperplasia and edema. Compared with the model group, the pathological morphological damage of lung in the GPR84 inhibitor group of low dose was improved to varying degrees. In the high dose group, a small amount of exudates such as neutrophils and red blood cells were observed, edema and hyperplasia were significantly improved (Fig. 2A). The wet/dry weight ratio of lung tissue reflects the degree of noncardiogenic pulmonary edema[12]. Compared with the control group, there was significant difference in the wet/dry weight ratio of lung tissue in LPS group. The wet/dry weight ratio of lung tissue in the GPR84 treatment group was markedly decreased when compared with control group (B).

GPR84 inhibitor changed the levels of inflammatory markers

ELISA results of bronchoalveolar lavage fluid showed that the expressions of TNF- α , IL-1 β and IL-6 in the LPS group were significantly higher than those in the control group 12 hours after LPS administration (Fig. 3A). But the expression of TNF- α , IL-1 β and IL-6 in BALF showed a significant decrease after GPR84 treatment. MPO is mainly synthesized and released by neutrophils, the activity of which in lung tissue is an important indicator to reflect neutrophil infiltration during the inflammatory process[13]. After LPS-induced ALI in mice for 12h, the MPO activity in lung tissues of mice in LPS group was increased compared with the control group (Fig. 3B). The MPO activity in the lung tissues of mice in LPS + GPR84 inhibitor group was lower than that in the model group. With the increase of GPR84 dose, MPO activity was significantly reduced. Cox-2 is an important downstream inflammatory mediator of NF- κ B pathway and plays an important role in the pathogenesis of ALI and is associated with disease severity[14]. After 12 hours of LPS-induced ALI in mice, the protein expressions of COX-2 and the ratio of p-IKK β /IKK β and p-p65/p65 in lung tissues of mice in LPS group were significantly increased compared with those in the control group (Fig. 3C). After the intervention of GPR84, these two proteins were significantly lower than those in the LPS group, indicating the suppression of GPR84 inhibitor for NF- κ B pathway.

GPR inhibitor reduced apoptosis levels

In this study, TUNEL assay was used to detect the effect of GPR inhibitor on apoptosis of lung tissue in mice with acute lung injury induced by LPS. Compared with the control group, the number of apoptotic cells in the lung tissues of the LPS group was significantly increased, indicating that LPS stimulation could promote cell apoptosis (Fig. 4A). The number of apoptotic cells in lung tissue of GPR inhibitor group was significantly lower than that of LPS group, indicating that GPR inhibitor could reduce the number of apoptotic cells in lung tissue of acute lung injury. Bcl-2 can form heterodimers with Bax, resulting in decreased cell permeability. When Bax is overexpressed, apoptosis can increase, while when Bcl-2 is overexpressed, its products can combine with Bax, thus inhibiting apoptosis. The results showed that the proteins promoting apoptosis were significantly reduced while the protein inhibiting apoptosis was increased (Fig. 4B).

GPR inhibitor suppressed the activation of MAPK

The levels of P38, ERK and JNK phosphorylated proteins in the lung tissues of mice in the model group was significantly increased compared to control group (Fig. 5A). The levels of above proteins in GPR inhibitor group were significantly reduced and the effect was dose-dependent when comparing with LPS group. Therefore, we predicted that the suppression of GPR inhibitor for inflammation and apoptosis could be derived from the suppression of AMPK signalling pathway. Next, the hypothesis was further validated in BEAS-2B cells treated by LPS. As shown in Fig. 5B, the treatment of BEAS-2B with GPR84 inhibitor resulted in increased cell viability. Moreover, similar effects of GPR84 inhibitor on AMPK signalling pathway were also observed in BEAS-2B cells with LPS stimulation (Fig. 5C).

The inhibition of MAPK signalling rescued the effects of GPR84 inhibitor on NF- κ B signalling

In addition to the treatment of LPS or GPR84 inhibitor, anisomycin (25 μ g/mL) as an activator of p38, or TPA (200 nM) as an activator of ERK was also used to treat BEAS-2B cells. The expression of TNF- α , IL-1 β and IL-6 was significantly increased in LPS group compared to control group. In addition, we found that anisomycin or TPA treatment could significantly counteract the effects of GPR84 inhibitor (Fig. 6A). NF- κ B P65 is a nuclear transcription factor present in the cytoplasm that participates in and regulates inflammatory responses. The ratio of phosphorylated protein to total protein was used to reveal the change of phosphorylated protein [15]. Compared with the normal group, the expression of NF- κ B p65 and I κ B β phosphorylated protein in LPS group was significantly up-regulated while GPR84 inhibitor could effectively reduce their expression (Fig. 6B). Intriguingly, anisomycin and TPA markedly reversed these effects of GPR84 inhibitor.

The inhibition of MAPK signalling reversed the effects of GPR84 inhibitor on apoptosis

As we aimed at investigating the action mechanism of GPR84 in apoptosis, we next set out experiments to analyze whether anisomycin or TPA affected the effects of GPR84 on apoptosis. We found that the effects of GPR84 inhibitor on apoptosis could also be reversed by anisomycin and TPA, suggesting that the suppression of apoptosis by GPR84 was mediated by MAPK signalling (Fig. 7A-B).

Discussion

LPS as the main component of the outer membrane of Gram-negative bacteria, has significant pathogenicity and is one of the important causes of ALI. In this study, LPS was used to induce ALI in vivo and vitro. We found that LPS induced the upregulation of GPR84 in mice and BEAS-2B cells and its inhibitor effectively ameliorated lung damage and increased survival rate. Furthermore, anisomycin or TPA treatment could reverse the effects of GPR84 inhibitor on inflammatory mediators and apoptosis in LPS-treated BEAS-2B cells. These findings indicated that the beneficial effects of GPR84 inhibitor in improving lung damage were relevant to reduced inflammation and apoptosis of lung tissue, which were mediated by MAPK signalling pathway.

Various lines of evidence demonstrated that blocking NF- κ B/MAPK has therapeutic efficacy on reducing acute lung injury [16, 17]. Experimental data from in vivo and vitro study of ALI showed that p38

MAPK was implicated in mediating NF- κ B signalling pathway, thereby affecting lung injury and macrophage activation[15]. The NF- κ B pathway is an important regulatory pathway for inflammation and immune response in the body and has been shown to play a key role in acute lung injury[18–20]. LPS induces the formation of inflammatory mediators through the NF- κ B pathway in ALI[21, 22]. In our work, anisomycin or TPA treatment was found to interfere with NF- κ B activation by GPR84 inhibitor, further revealing that GPR84 inhibitor could suppressed MAPK signalling to involve in NF- κ B activation. In our work, the treatment of IL-33 markedly elevated the mRNA and protein of GPR84 compared with LPS alone in vivo and vitro. Many researches have reported that IL-33 activated AMPK components and modulated NF- κ B pathway [23–26]. Therefore, GPR84 could implicate in the regulation of IL-33 for MAPK signalling pathway.

To sum up, GPR84 inhibitor significantly alleviated lung inflammation and apoptosis in LPS-induced acute lung injury (ALI) mice and BESA-2B and played a protective role in lung injury of ALI. The mechanism was related to inhibiting the activation of MAPK/NF- κ B signaling pathway.

Declarations

Conflict of interest

The authors declare no conflict of interest.

Ethics approval and consent to participate

The manuscript was approved by the Ethics Committee of The First Affiliated Hospital of Harbin Medical University.

Consent for publication

The Author agrees to publication in the Inflammation Journal and also to publication of the article in English.

Availability of data and materials

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

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Authors' contributions

He Lv, Ting Wang and Yan Zou conceived and designed the study, collected, analysed and interpreted the data, and revised the manuscript. LJ Ting Wang the manuscript. All authors read and approved the final manuscript.

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Figures

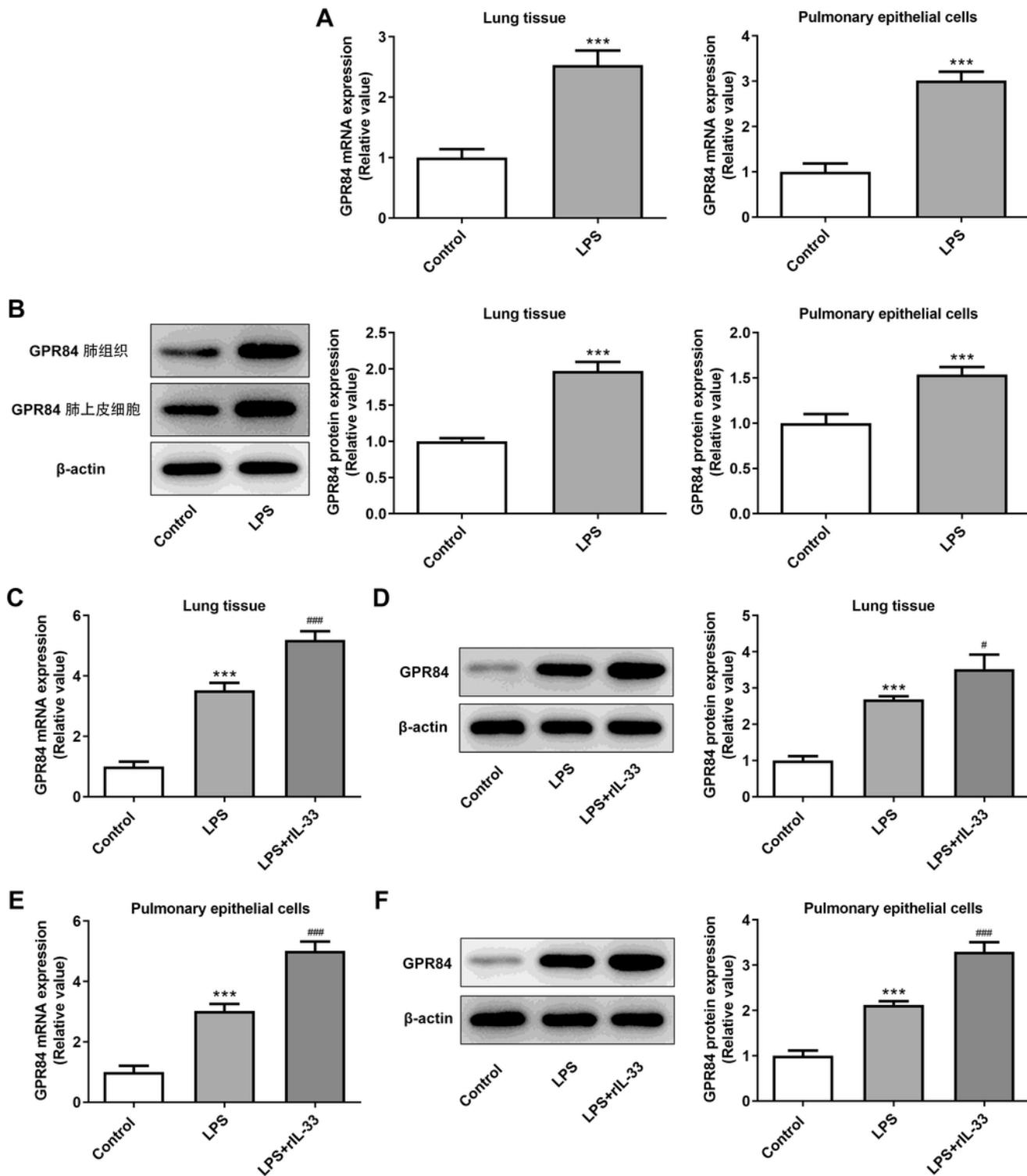


Figure 1

The detection of GPR84 mRNA through qPCR (A) and protein levels through Western blot (B). The induction of GPR84 expression in lung tissue (C-D) and pulmonary epithelial cells (E-F). *** $p < 0.001$ Vs Control group. # $p < 0.05$, ### $p < 0.001$ Vs LPS group.

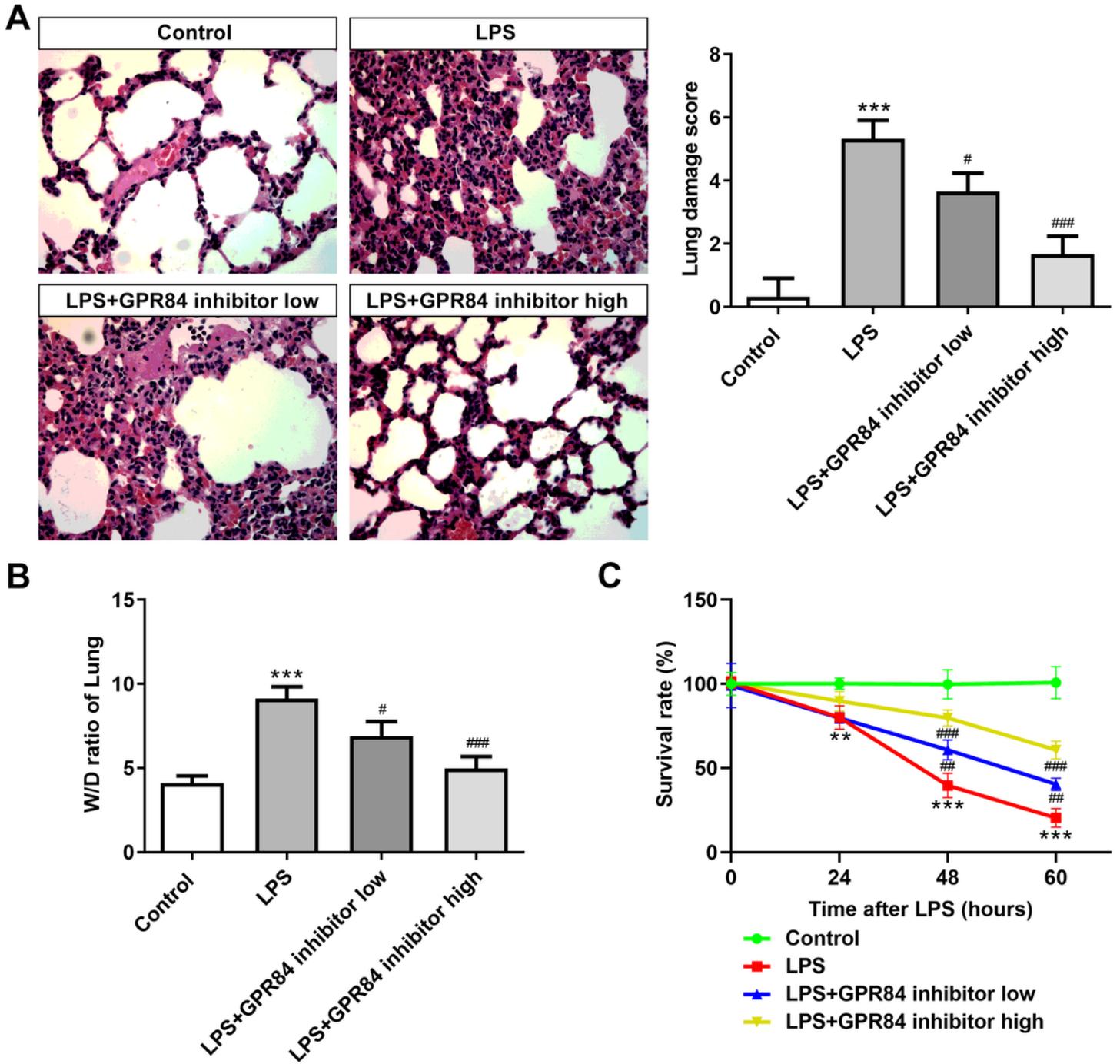


Figure 2

The HE staining (A) and the ratio of wet weight to dry weight of lung tissue in each group (B). ^{***} $p < 0.001$ Vs Control group. [#] $p < 0.05$, ^{###} $p < 0.001$ Vs LPS group.

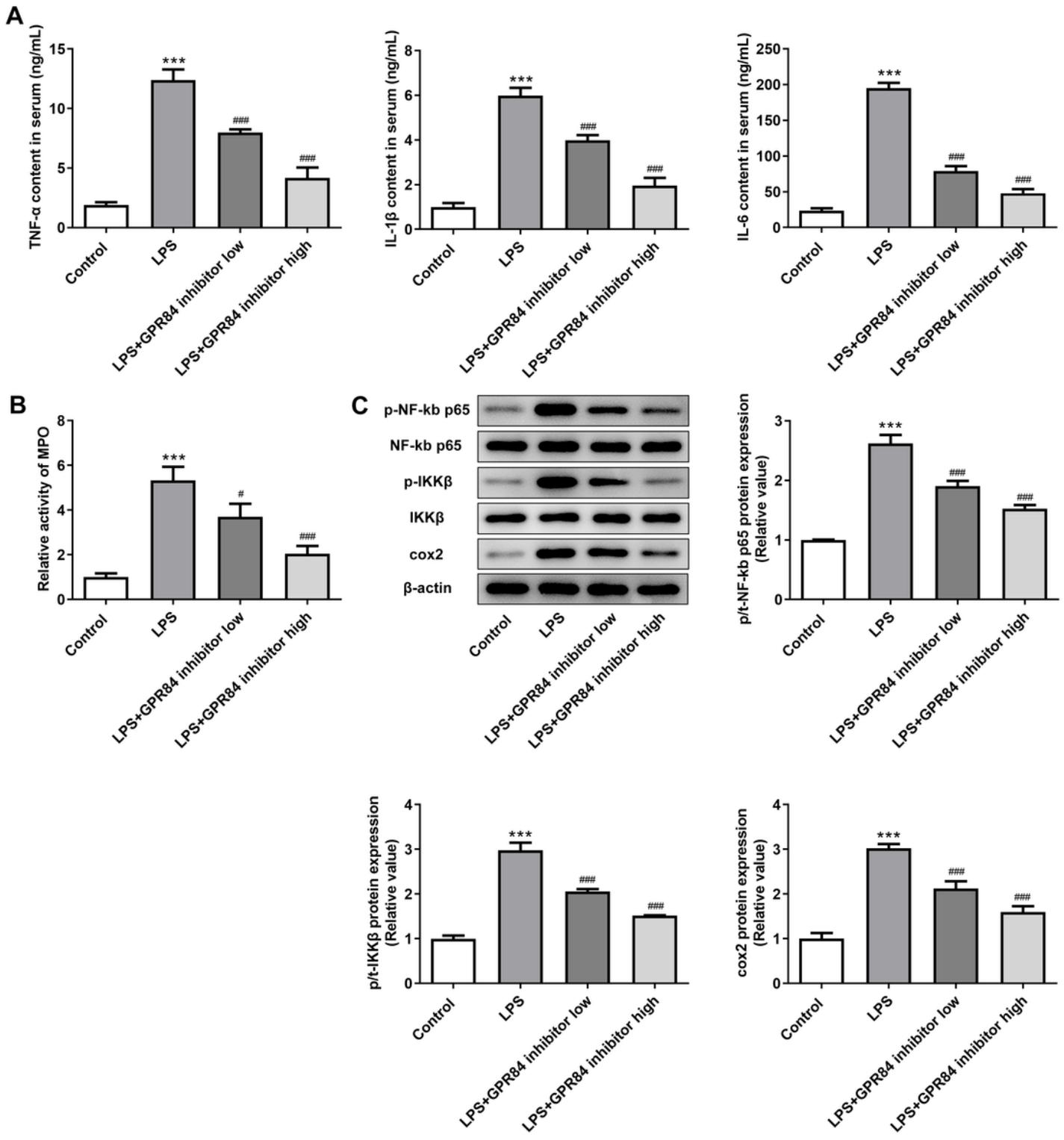


Figure 3

Expression of TNF-α, IL-1β and IL-6 in BALF of mice in each group (A), MPO levels and the phosphorylation levels of NF-κB P65 and IKKβ (B), as well as COX-2 protein expression (C) of mice in each group. *** $p < 0.001$ Vs Control group. # $p < 0.05$, ### $p < 0.001$ Vs LPS group.

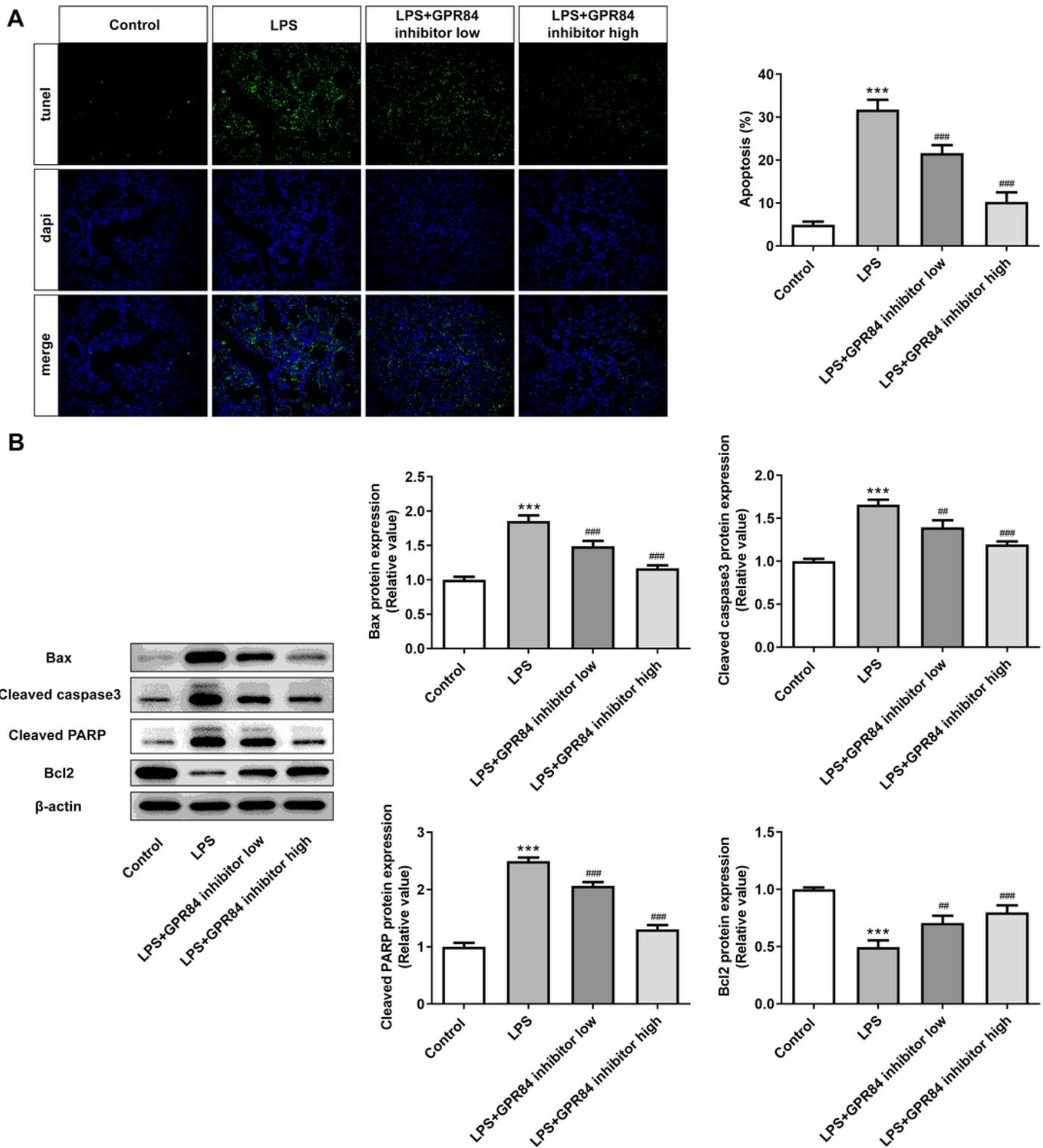


Figure 4

The Tunel staining (A) and analysis of Western blot for the protein levels of Bax, cleaved caspase3, cleaved PARP and Bcl-2. ^{***} $p < 0.001$ Vs Control group. ^{##} $p < 0.01$, ^{###} $p < 0.001$ Vs LPS group.

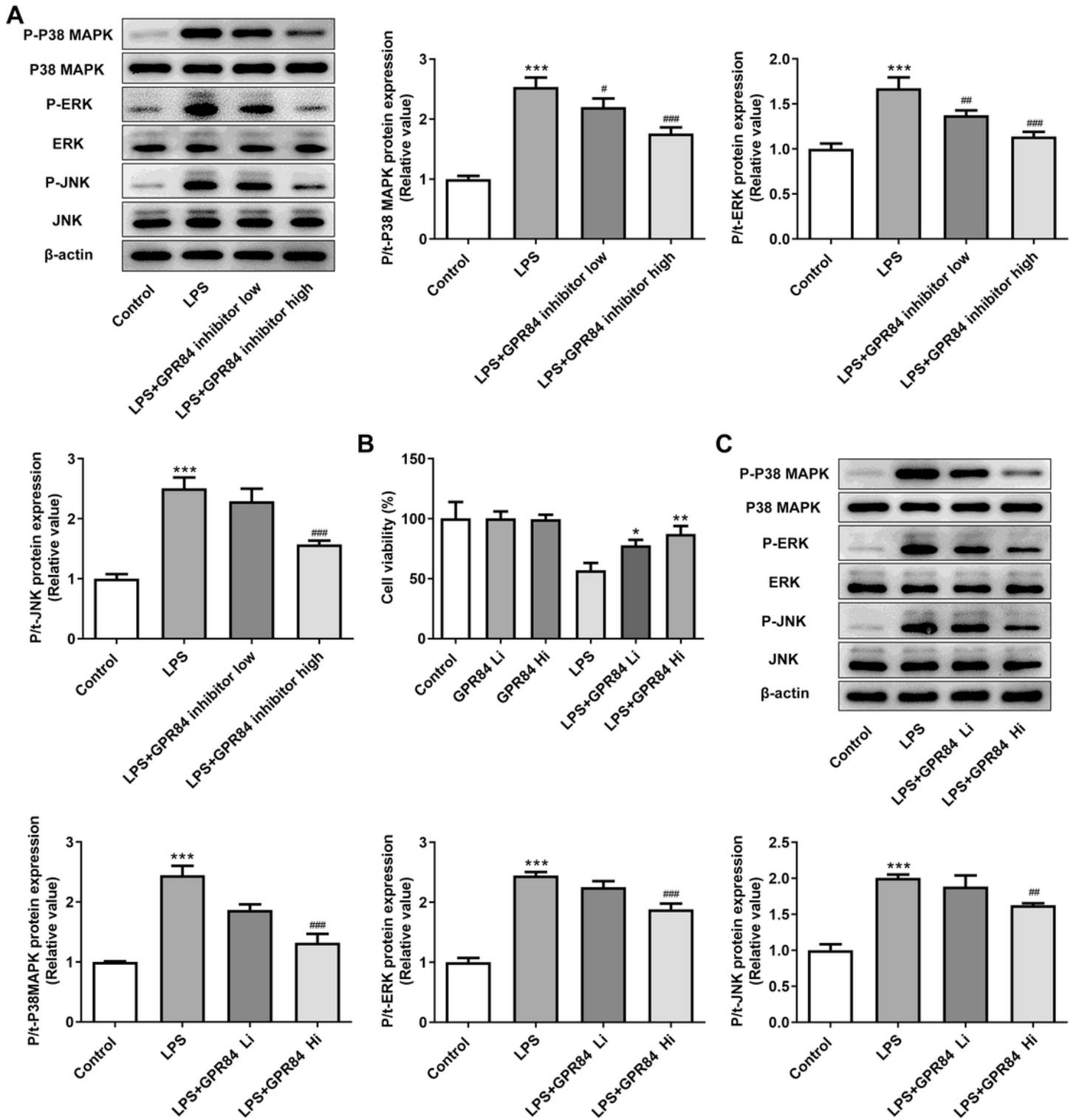


Figure 5

The effects of GPR84 inhibitor on the expression of key proteins in MAPK pathway in lung tissues of ALI mice (A) and, on cell viability * $p < 0.05$ and ** $p < 0.01$ Vs LPS group (B), and in MAPK pathway (C) in BEAS-2B cells treated by LPS. *** $p < 0.001$ Vs Control group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ Vs LPS group.

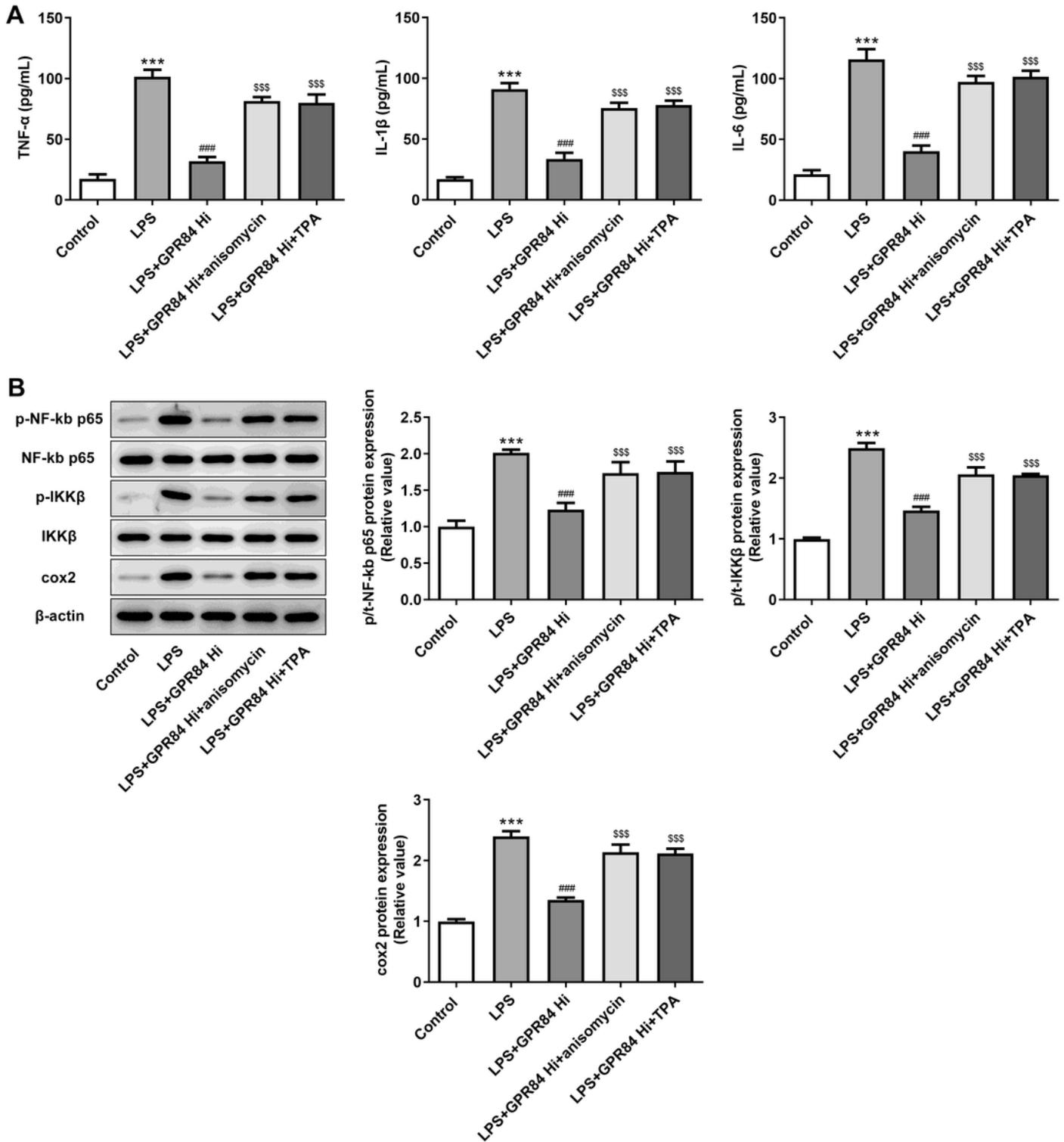


Figure 6

The detection of TNF- α , IL-1 β and IL-6 levels through ELISA assay (A) and the analysis of NF- κ B signalling through Western blotting. *** p <0.001 Vs Control group. ### p <0.001 Vs LPS group. \$\$\$ p <0.001 Vs LPS+GPR84 Hi.

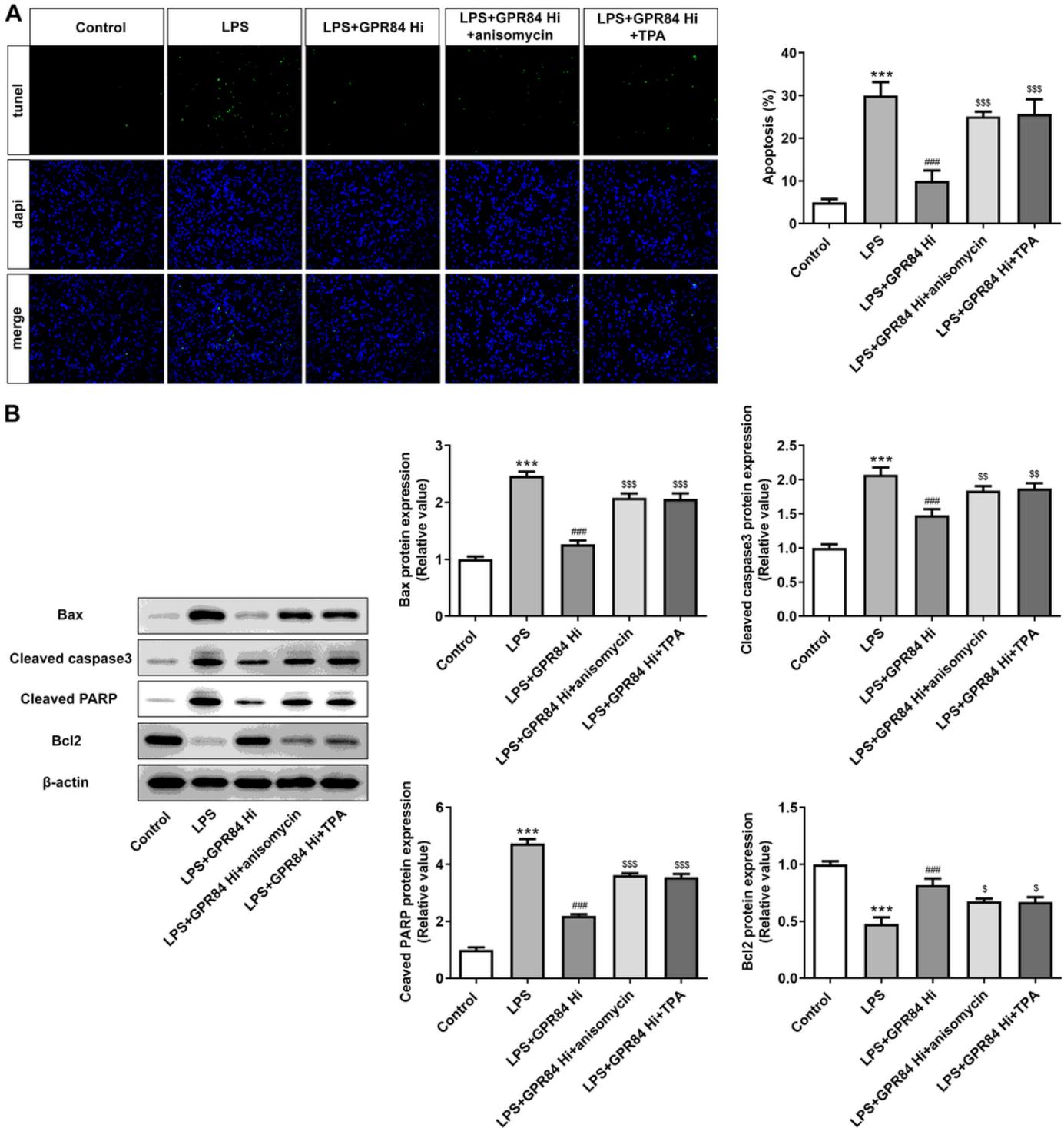


Figure 7

The detection of cell apoptosis levels through TUNEL assay (A) and the expression analysis of apoptosis mediators through Western blot. *** $p < 0.001$ Vs Control group. ### $p < 0.001$ Vs LPS group. \$ $p < 0.05$,

$p < 0.01$,

\$ $p < 0.001$ Vs LPS+GPR84 Hi.