

# Dapk1 promoted inflammation of infantile pneumonia by p38MAPK/NF- $\kappa$ B signaling pathway

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## Research

**Keywords:** Dapk1, p38MAPK, NF- $\kappa$ B, infantile pneumonia

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# Abstract

**Background:** The present study was designed to investigate the function of Death associated protein kinase 1 (DAPK1) in infantile pneumonia and explore the potential mechanism of the actions.

**Methods:** Male C57BL/6 mice were injected with 2 mg/kg of LPS for the mice model of infantile pneumonia. A549 cell were treated with 100 ng/ml of LPS for vitro model of infantile pneumonia. Dapk1 mRNA and protein expressions in 6, 12 or 24 h after induction model of mice.

**Results:** Dapk1 gene increased inflammation in vitro model through activation of p38MAPK-mediated NF- $\kappa$ B expression. The inhibition of p38MAPK or NF- $\kappa$ B reduced the pro-inflammation effects of DAPK1 in infantile pneumonia.

**Conclusions:** Our study demonstrates that Dapk1 promoted inflammation of infantile pneumonia by p38MAPK/NF- $\kappa$ B signaling pathway, may be achieved inflammation by activation of p38MAPK/NF- $\kappa$ B signaling pathway

## Introduction

Mycoplasmal pneumonia is a respiratory infection caused by mycoplasma pneumoniae, which is clinically manifested by fever, cough, sputum expectoration, anorexia, headache, sore throat, substernal pain and chills (1). The more atypical clinical symptoms are generally detected in younger children, often accompanied with multi-system and multi-organ functional damage (2). Infantile mycoplasmal pneumonia is one of the main causes of respiratory infection in children (2). If not treated in time, it may cause bronchiectasis, atelectasis and pulmonary interstitial fibrosis, and even death in severe cases (3). At present, the etiology of infantile pneumonia is not completely clear, which might be associated with the humoral and cellular immunity of the children (3). Therefore, anti-infection and symptomatic treatment are the main therapeutic approaches.

NF- $\kappa$ B and p38 MAPK signaling pathways play important roles in the inflammatory process (4, 5). Both of them can promote the occurrence and development of inflammatory response in lung tissue by regulating multiple inflammatory factors, such as IL-12, IL-13 and TNF- $\alpha$  and promoting the infiltration of inflammatory cells (4, 5). Many studies have shown that NF- $\kappa$ B and p38 MAPK signaling pathways are involved in pneumonia caused by Streptococcus pneumoniae (6, 7). However, there are relatively rare studies on the role of NF- $\kappa$ B and p38 MAPK signaling pathways on MP-infected pneumonia (8).

Death associated protein kinase 1 (DAPK1) is a serine/threonine protein kinase regulated by calmodulin (CaM), involved in a variety of pathophysiological processes in the body (9). Previous studies have shown that DAPK1 is one of the positive regulators of apoptosis, which is widely involved in apoptosis induced by multiple pathways (10–12). Recent studies have also found that in addition to regulating apoptosis and autophagy, DAPK1 also plays a vital role in a series of inflammation regulation (10–12).

The present study was designed to investigate the function of DAPK1 in infantile pneumonia and explore the potential mechanism of the actions.

## Materials And Methods

### Animals and vivo model

Male C57BL/6 mice (1 week, 4–5 g, n = 50) were injected with 50 mg/kg pentobarbital sodium pentobarbital sodium and then were injected with 2 mg/kg of LPS (Sigma-Aldrich Merck KGaA). C57BL/6 mice were randomly assigned to one of two groups: Sham (n = 10), 3 h of IP group (n = 10), 6 h of IP group (n = 10), 12 h of IP group (n = 10) and 24 h of IP group (n = 10). Mice were sacrificed using decollation under 50 mg/kg pentobarbital sodium. All the animal experiments were conducted under the guidelines issued by the The Affiliated Changzhou NO.2 People's Hospital of Nanjing Medical University Institutional Animal Care and Use Committee. The protocols for the experiments in this study were reviewed and approved by University Research Committee of The Affiliated Changzhou NO.2 People's Hospital of Nanjing Medical University

### Gene microarray hybridization.

Total RNA was labelled using cyanine-5-cTP and hybridized to the SurePrint and G3 Mouse Whole Genome GE 8 × 60 K Microarray G4852A platform. Images were quantified and feature extracted using Agilent Feature Extraction Software (v.A.10.7.3.1; Agilent Technologies, Inc.).

### Cell Culture and Vitro model

Human lung cancer cell line A549 cell was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). A549 cell was maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) in a humidified incubator at 37 °C and 5% CO<sub>2</sub>. Dapk1, siDapk1, NF-κB, siNF-κB and negative mimics were transfected into cells (1 × 10<sup>5</sup> cell/ml) using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) for 48 h. After transfection with plasmids, cells were treated with 100 ng/ml of LPS for 6 h.

### Histological Examination

Lung tissue samples after mice sacrificed were collected and fixed with 4% paraformaldehyde for 24 h at room temperature. All experiments have been approved by the Ethics Committee of The Affiliated Changzhou NO.2 People's Hospital of Nanjing Medical University. Lung tissue samples fixed with paraformaldehyde were paraffin-embedded. Lung tissue samples were cut into 5 μm sections using a paraffin slicing machine and stained with hematoxylin and eosin. Lung tissues were observed under light microscopy (magnification, × 100; BH3-MJL; Olympus Corporation, Tokyo, Japan).

### Quantitative Real-Time PCR Detection

Total RNA was extracted from tissue sample or cells samples by using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA). Taqman MicroRNA Reverse Transcription Kit and Taqman Universal

Master Mix II with TaqMan MRNA Assay (Applied Biosystems, Foster City, CA, USA) were used for testing the gene expression level.

### **ELISA Assay**

Cell samples and serum samples were collected and then the concentrations of TNF- $\alpha$ , IL-6, IL-1 $\beta$  and IL-18 levels were measured using *ELISA kits*

### **Western blot analysis**

Proteins were extracted by using radioimmunoassay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China) and were quantified by the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Total proteins were loaded onto 10% SDS-PAGE, separated by electrophoresis and transferred to PVDF membranes. Membranes were blocked by 5% skimmed milk in PBS/Tween 20 for 1 h with shaking and then incubated overnight at 4 °C with the specific primary antibodies Dapk1 (1:1000, Cell Signaling Technology), p-p38MAPK (1:1000, Cell Signaling Technology), p38MAPK (1:500, Santa Cruz Biotechnology), NF- $\kappa$ B (1:500, Santa Cruz Biotechnology) and  $\beta$ -Actin (1:5000, Santa Cruz Biotechnology). The membranes were incubated with the secondary antibodies conjugated with HRP for 1 h after washing with TBST for 15 min. Finally, images were captured using Fujifilm LAS-4000 mini (Fujifilm, Tokyo, Japan).

### **Immunofluorescent staining.**

After experiment, A549 cell was fixed with 4% paraformaldehyde for 15 min at room temperature and then incubated with using 0.25% Triton X100 for 15 min. A549 cell was incubated with p-p38 MAPK (1:100) at 4°C overnight after blocking with 5% BSA for 1 h. A549 cell was incubated with goat anti-rabbit IgG-cFL 555 antibody (1:100) for 2 h at room temperature and stained with DAPI for 15 min and washed with PBS for 15 min. The images of A549 cell obtained using a Zeiss Axioplan 2 fluorescent microscope (Carl Zeiss AG, Oberkochen, Germany).

### **Statistical Analysis**

All experiments were repeated at least three times. P-values were calculated using two-tailed Student's t test between two groups, or one-way analysis of variance for more than two groups. Statistical analyses were performed by using SPSS 19.0 statistical software. P-value of < 0.05 was considered to indicate a statistically significant result.

## **Results**

### **Dapk1 expression in mice of infantile pneumonia**

Firstly, gene chip was used to analyze the changes expression of pro-inflammation gene in mice of infantile pneumonia. As shown in Fig. 1A, Dapk1 expression maybe one pro-inflammation gene and was up-regulated in mice of infantile pneumonia as 24 h after induction model. Next, we found that Dapk1

mRNA and protein expressions in 6, 12 or 24 h after induction model, compared to sham mice (Fig. 1B-1D).

### **Dapk1 gene increased inflammation in vitro model**

Then, we used vitro model to analyze the effects of Dapk1 gene on infantile pneumonia. Over-expression of Dapk1 increased TNF- $\alpha$ , IL-6, IL-1 $\beta$  and IL-18 levels in vitro model, compared to negative group (Fig. 2A-2D). Down-regulation of Dapk1 reduced TNF- $\alpha$ , IL-6, IL-1 $\beta$  and IL-18 levels in vitro model, compared to negative group (Fig. 2E-2H). These results showed that Dapk1 gene increased inflammation in vitro model of infantile pneumonia.

### **Dapk1 promotes p38MAPK-mediated NF- $\kappa$ B Expression**

Gene chip was used to analyze inflammation signaling pathway by regulation of Dapk1 in infantile pneumonia. As shown in Fig. 3A-3B, p38MAPK/NF- $\kappa$ B expression signaling pathway may a target spot for the function of Dapk1 in infantile pneumonia. Over-expression of Dapk1 induced Dapk1, p-p38MAPK and NF- $\kappa$ B protein expression in vitro model of infantile pneumonia, compared to negative group (Fig. 3C-3F). Down-regulation of Dapk1 suppressed p-p38MAPK and NF- $\kappa$ B protein expression in vitro model of infantile pneumonia, compared to negative group (Fig. 3C-3F). IF showed over-expression of Dapk1 induced p-p38MAPK expression in vitro model of infantile pneumonia, compared to negative group (Fig. 3G). Down-regulation of Dapk1 suppressed Dapk1, p-p38MAPK and NF- $\kappa$ B protein expression in vitro model of infantile pneumonia, compared to negative group (Fig. 3F, 3H-3J).

### **The regulation of p38MAPK participated in the effects of Dapk1 in infantile pneumonia**

The study evaluated the role of p38MAPK in the function of Dapk1 in infantile pneumonia. Dehydrocorydaline nitrate (10 nM, p38MAPK agonist, MedChemExpress) induced p-p38MAPK and NF- $\kappa$ B protein expressions in vitro model following with down-regulation of Dapk1 (Fig. 4A-4C). Then, the activation of p38MAPK also increased the TNF- $\alpha$ , IL-6, IL-1 $\beta$  and IL-18 levels in vitro model following with down-regulation of Dapk1 (Fig. 4D-4G).

p38 MAPK-IN-1 (20 nM, 38MAPK inhibitor, MedChemExpress) suppressed p-p38MAPK and NF- $\kappa$ B protein expressions in vitro model following with up-regulation of Dapk1 (Fig. 5A-5C). Then, the inhibition of p38MAPK also reduced the TNF- $\alpha$ , IL-6, IL-1 $\beta$  and IL-18 levels in vitro model following with up-regulation of Dapk1 (Fig. 5D-5G).

### **The regulation of NF- $\kappa$ B participated in the effects of Dapk1 in infantile pneumonia**

Lastly, si-NF- $\kappa$ B suppressed the up-regulation of Dapk1 on NF- $\kappa$ B protein expression, and the TNF- $\alpha$ , IL-6, IL-1 $\beta$  and IL-18 levels in vitro model, compared with up-regulation of Dapk1 group (Fig. 6). NF- $\kappa$ B plasmid induced NF- $\kappa$ B protein expression, and the TNF- $\alpha$ , IL-6, IL-1 $\beta$  and IL-18 levels in vitro model by down-regulation of Dapk1, compared with down-regulation of Dapk1 group (Fig. 7).

## Discussion

Infantile pneumonia is a common pediatric disorder in lung tissues, which occurs at all ages of children, especially in infants and young children (13). The common pathogens mainly bacteria, viruses and mycoplasma. Anti-infection and symptomatic treatment are used by Western Medicine. And most children can be cured within approximately one week (14). However, a considerable number of children, especially infants under 6 months of age, often have prolonged illness and delayed healing, such as persistent rales, phlegm in the throat, constant sputum and coughing, excessive sweating, poor appetite and loose stools (15). According to Western Medicine, only anti-infection and symptomatic treatment can be continued, which generally fail to significantly relieve the condition. In addition, some children have worsening diarrhea, causing microbiota imbalance, and even nosocomial cross infection. The present study demonstrated that Dapk1 mRNA and protein expressions in 6, 12 or 24 h after induction model. Li et al. showed that Dapk1 increased inflammation Dapk1 improves inflammation in LPS-induced acute lung injury via p38MAPK/NF- $\kappa$ B signaling pathway (16). At this stage, Dapk1 may regulated inflammation in infantile pneumonia.

p38, a member of the MAPK family, is mainly involved in apoptosis and differentiation, cell inflammation and oxidative stress (5). p38 is a key factor of the MAPK signaling pathway (5, 17). The activation of p38 MAPK can affect the activity of transcription factors, thereby regulating the inflammatory response (5, 17). Studies have reported that the p38 MAPK signaling pathway can promote the phosphorylation and degradation of I- $\kappa$ B $\alpha$ , thereby activating the NF- $\kappa$ B pathway (18)(19). Furthermore, this study demonstrated that Dapk1 promotes p38MAPK-mediated NF- $\kappa$ B expression to increase inflammation in vitro model of infantile pneumonia. Wu et al. reveal that tumor suppressor Dapk1 inhibits necroptosis by p38 MAPK activation (20).

NF- $\kappa$ B is an important nuclear transcription factor that regulates the immune response and inflammatory pathways. Under the normal state of the body, NF- $\kappa$ B binds to I- $\kappa$ B $\alpha$  in an inactive state in the plasma (14) (21)(22). Under the stimulation of pathogenic factors, I- $\kappa$ B $\alpha$  is phosphorylated and degraded, subsequently separated from the NF- $\kappa$ B heterodimer to expose the nuclear localization signal of p50 protein to activate NF- $\kappa$ B (21). Activated NF- $\kappa$ B transfers to the nucleus and combines with the target gene  $\kappa$ B to induce the expression of inflammatory factors, which is an important transcription factor to cause inflammatory response caused in infantile pneumonia (22). The present study demonstrated that the inhibition of p38 MAPK or NF- $\kappa$ B reduced the effects of Dapk1 on inflammation of infantile pneumonia. Wu et al. suggest that DAPK1 modulates a curcumin-induced G2/M arrest and by NF- $\kappa$ B activation (23). These results showed that Dapk1 regulated p38 MAPK/ NF- $\kappa$ B pathway to promote inflammation in infantile pneumonia.

In conclusion, the present study demonstrates that Dapk1 gene and protein expression was up-regulated in infantile pneumonia. Dapk1 induced p38MAPK/NF- $\kappa$ B signaling pathway to promote inflammation. These results reveal a novel pro-inflammation of Dapk1 in infantile pneumonia, suggesting that it may be a promising therapeutic target for the treatment of IP.

# Declarations

## Ethics approval and consent to participate

This study was approved by the Ethics committee of the Affiliated Changzhou NO.2 People's Hospital of Nanjing Medical University .

## Consent for publication

Not applicable.

## Availability of data and material

Not applicable.

## Funding

None.

## Competing interests

There are no potential conflicts of interest to disclose.

## Acknowledgements

Not applicable.

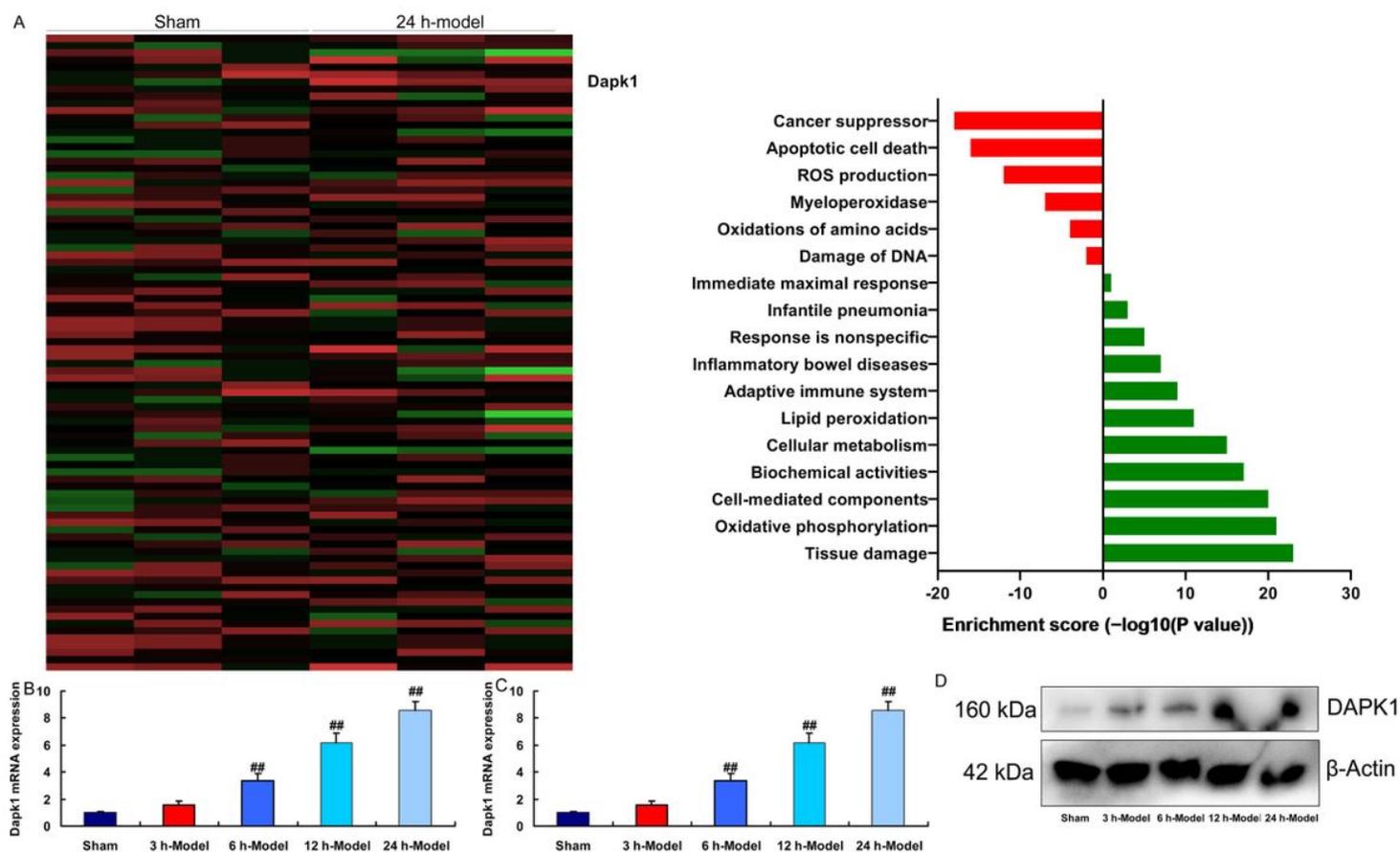
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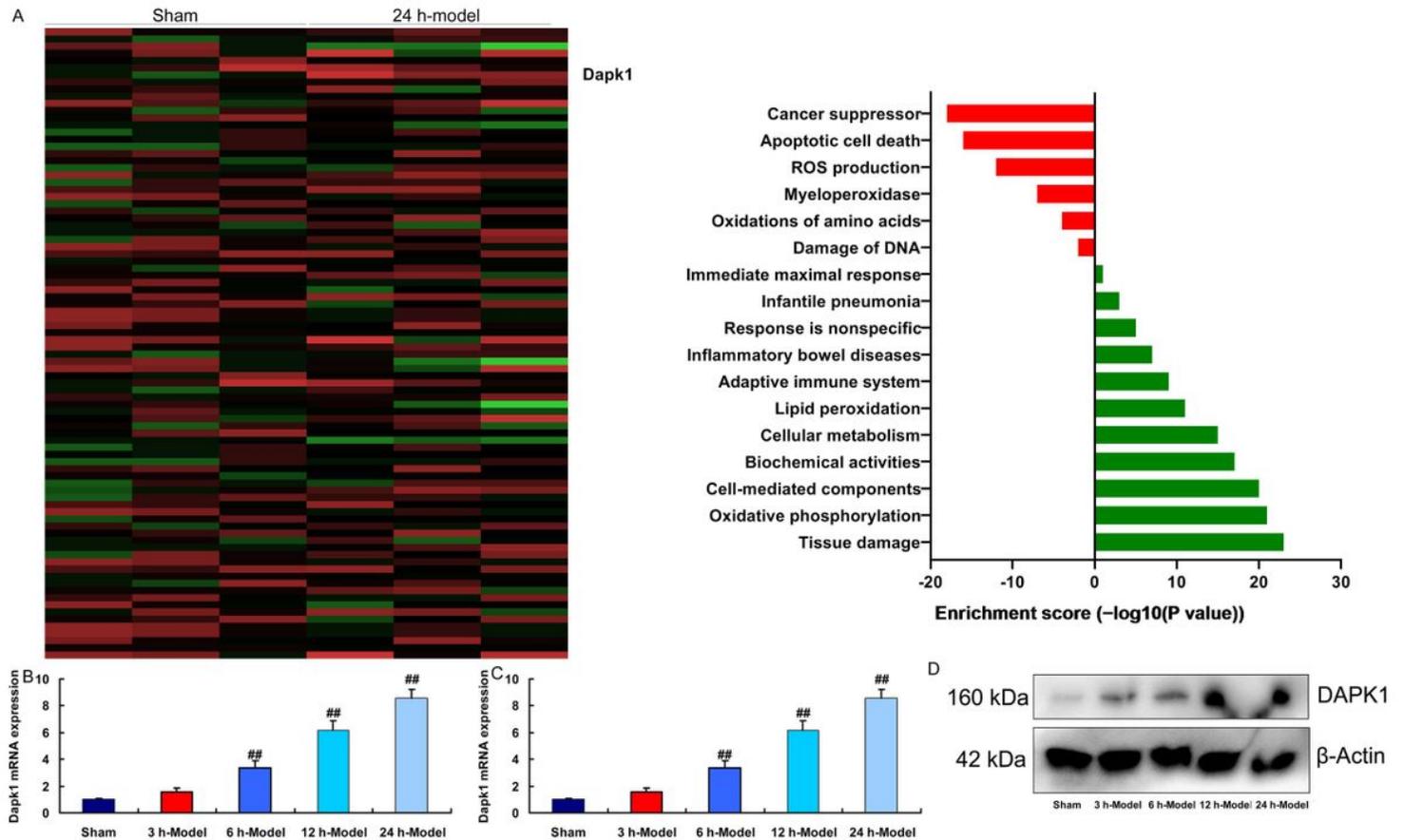
## Figures



**Figure 1**

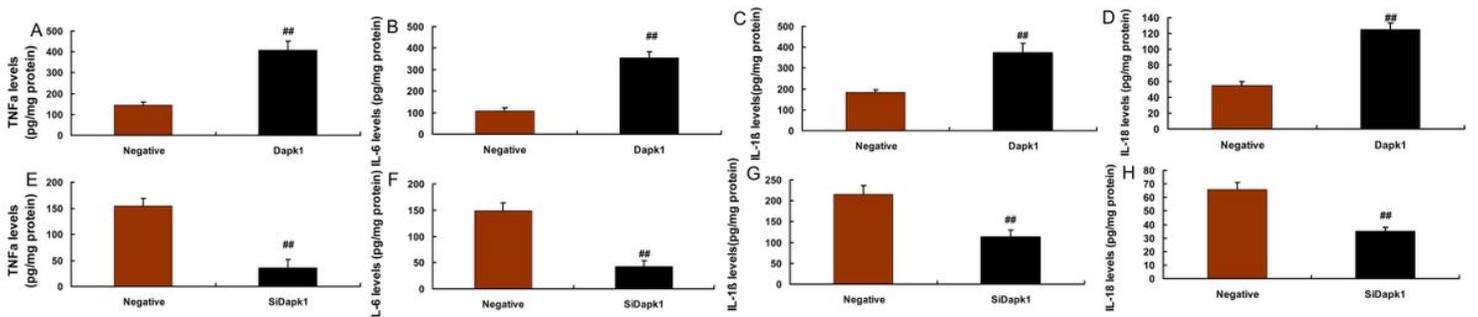
Dapk1 expression in mice of infantile pneumonia Heat map and microarray data (A), Dapk1 mRNA (B) and protein (C and D) expressions in 6, 12 or 24 h after induction model. Sham, sham control group; 3 h-Model, LPS induced infantile pneumonia for 3 h; 6 h-Model, LPS induced infantile pneumonia for 6 h; 12 h-Model, LPS induced infantile pneumonia for 12 h; 24 h-Model, LPS induced infantile pneumonia for 24 h.

h-Model, LPS induced infantile pneumonia for 12 h; 24 h-Model, LPS induced infantile pneumonia for 24 h. ## $p < 0.01$  compared with sham control group.



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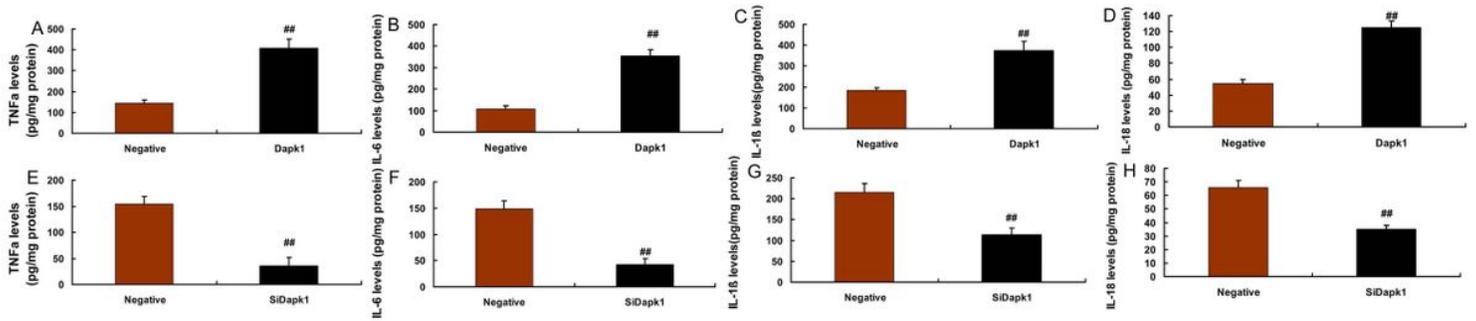
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**Figure 2**

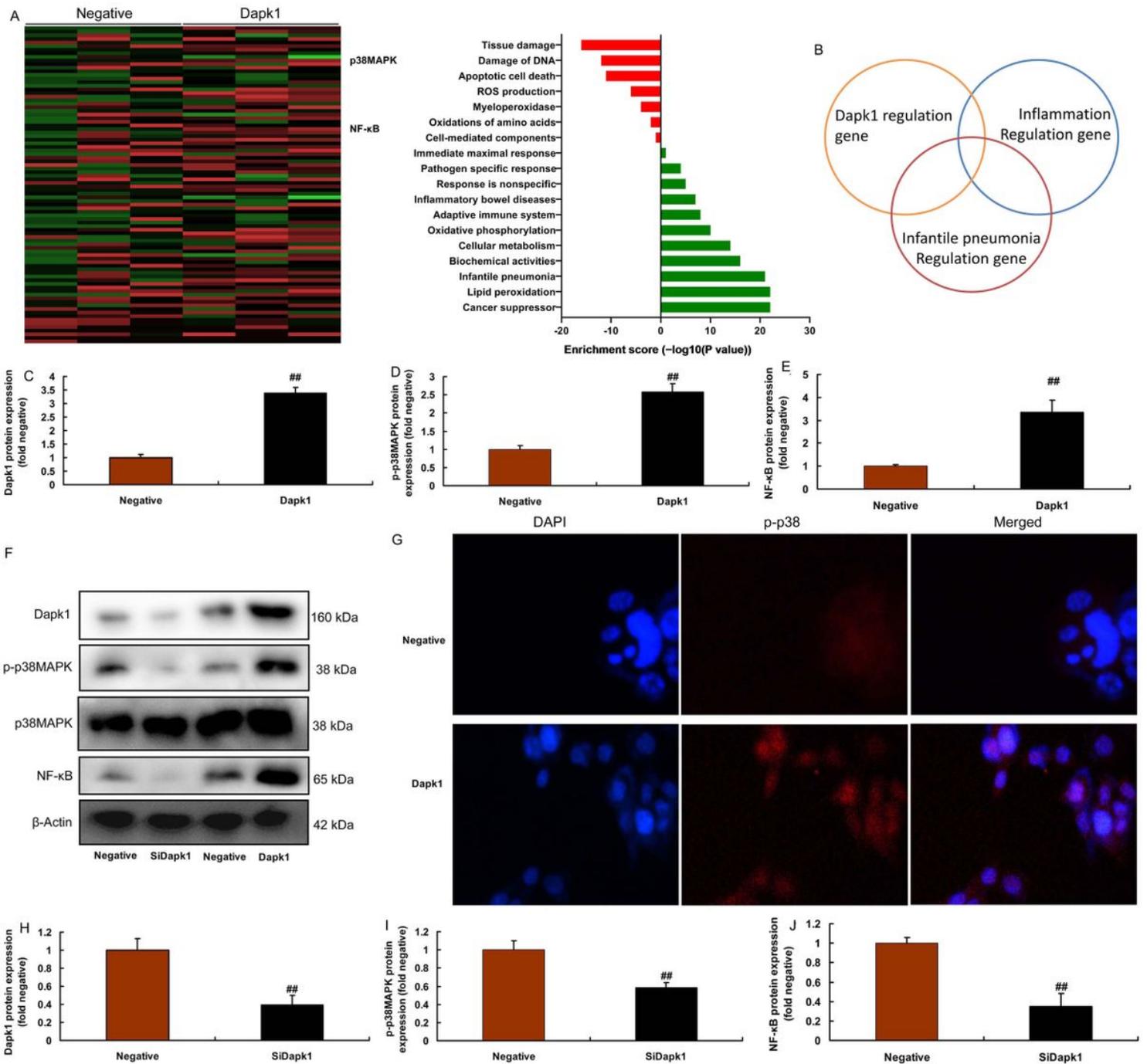
Dapk1 gene increased inflammation in vitro model TNF- $\alpha$  (A), IL-6 (B), IL-1 $\beta$  (C) and IL-18 (D) levels in vitro model of infantile pneumonia by over-expression of Dapk1; TNF- $\alpha$  (E), IL-6 (F), IL-1 $\beta$  (G) and IL-18

(H) levels in vitro model of infantile pneumonia by down-regulation of Dapk1. Negative, negative mimic group; Dapk1, over-expression of Dapk1 group; siDapk1, down-regulation of Dapk1 group.##p<0.01 compared with negative mimic group.



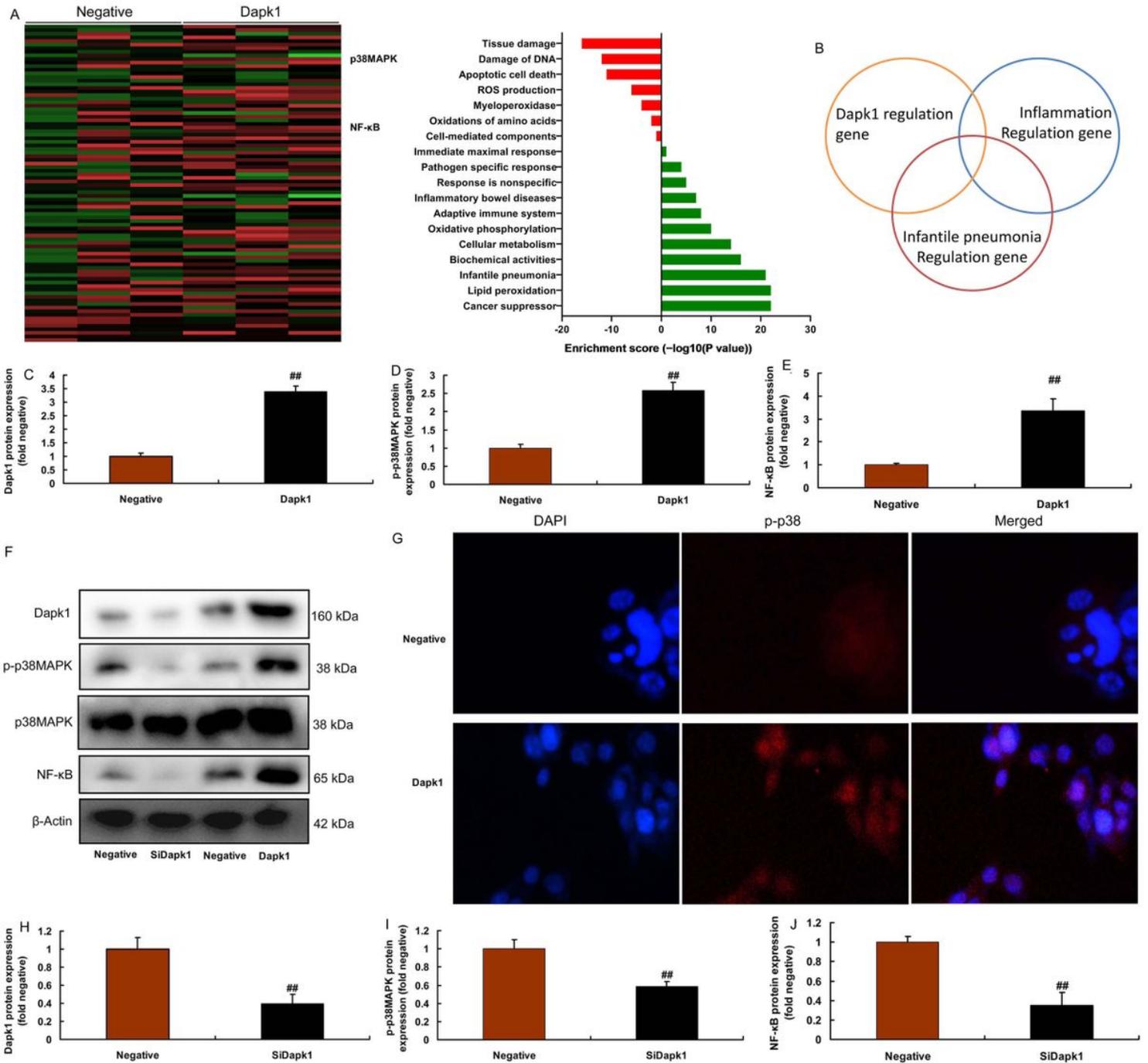
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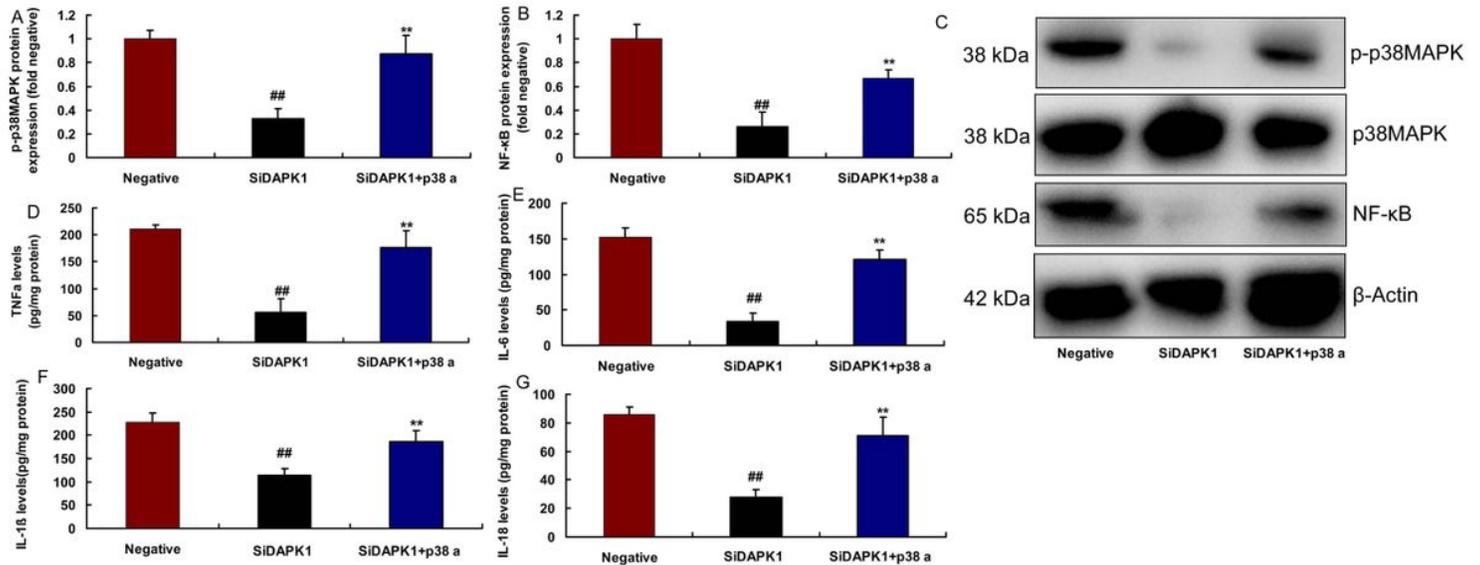
**Figure 3**

Dapk1 promotes p38MAPK-mediated NF-κB Expression Heat map and microarray data (A), interpretation of result (B), Dapk1, p-p38MAPK and NF-κB protein expression in vitro model by over-expression of Dapk1 (C, D, E and F); p-p38MAPK protein expression (immunofluorescence, G), Dapk1, p-p38MAPK and NF-κB protein expression in vitro model by down-regulation of Dapk1 (F, H, I and J). Negative, negative mimic group; Dapk1, over-expression of Dapk1 group; siDapk1, down-regulation of Dapk1 group.##p<0.01 compared with negative mimic group.



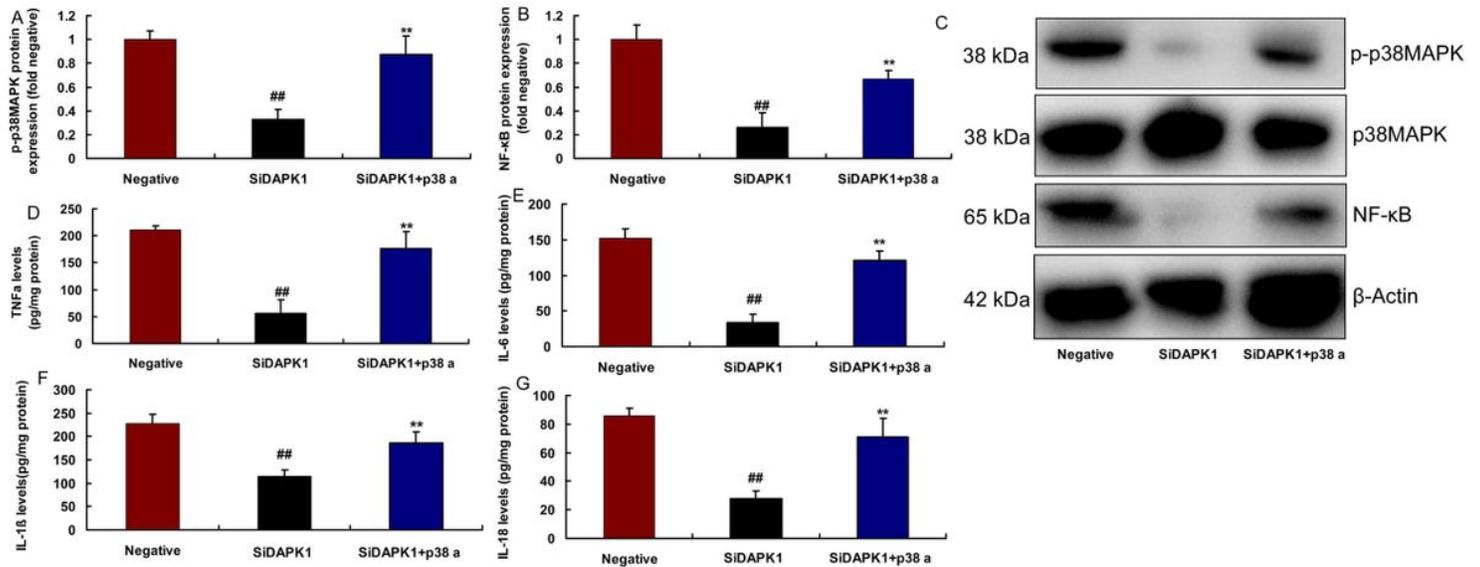
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Dapk1 promotes p38MAPK-mediated NF-κB Expression Heat map and microarray data (A), interpretation of result (B), Dapk1, p-p38MAPK and NF-κB protein expression in vitro model by over-expression of Dapk1 (C, D, E and F); p-p38MAPK protein expression (immunofluorescence, G), Dapk1, p-p38MAPK and NF-κB protein expression in vitro model by down-regulation of Dapk1 (F, H, I and J). Negative, negative mimic group; Dapk1, over-expression of Dapk1 group; siDapk1, down-regulation of Dapk1 group.## $p < 0.01$  compared with negative mimic group.



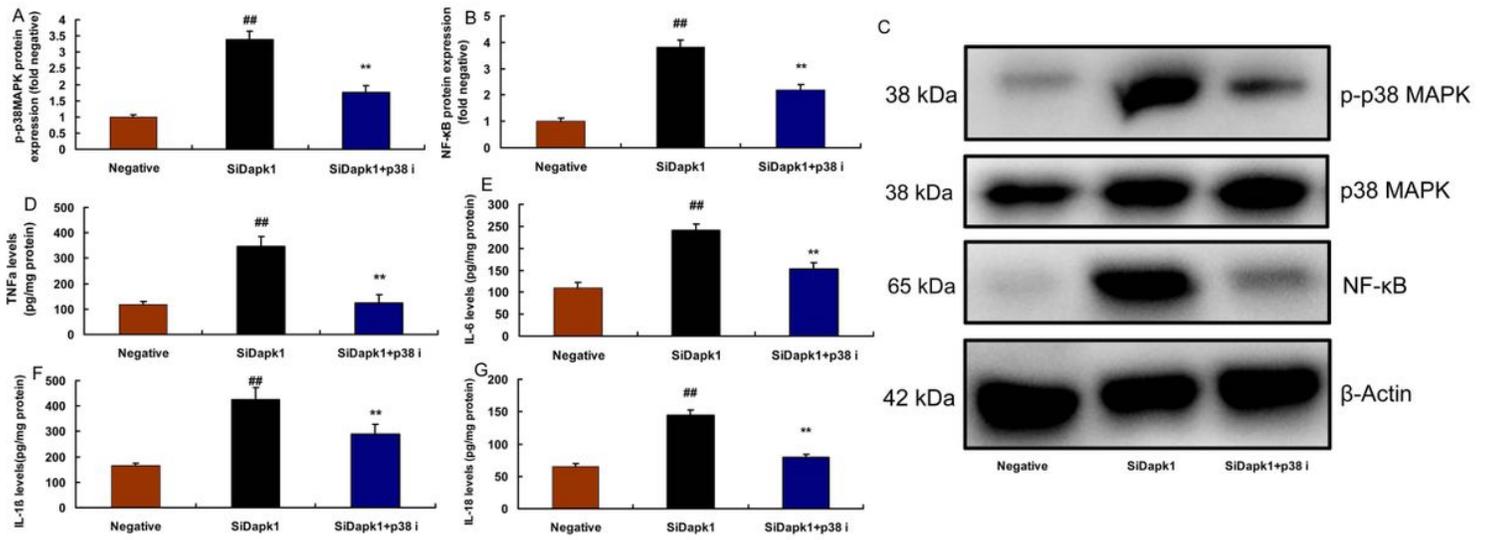
**Figure 4**

The induction of p38MAPK participated in the effects of Dapk1 in infantile pneumonia p-p38MAPK and NF-κB protein expression (A, B and C), TNF-α (D), IL-6 (E), IL-1β (F) and IL-18 (G) levels. Negative, negative mimic group; siDapk1, down-regulation of Dapk1 group; siDapk1+p38 a, down-regulation of Dapk1 and p38 agonist group. ##p<0.01 compared with negative mimic group; \*\*p<0.01 compared with down-regulation of Dapk1 group.



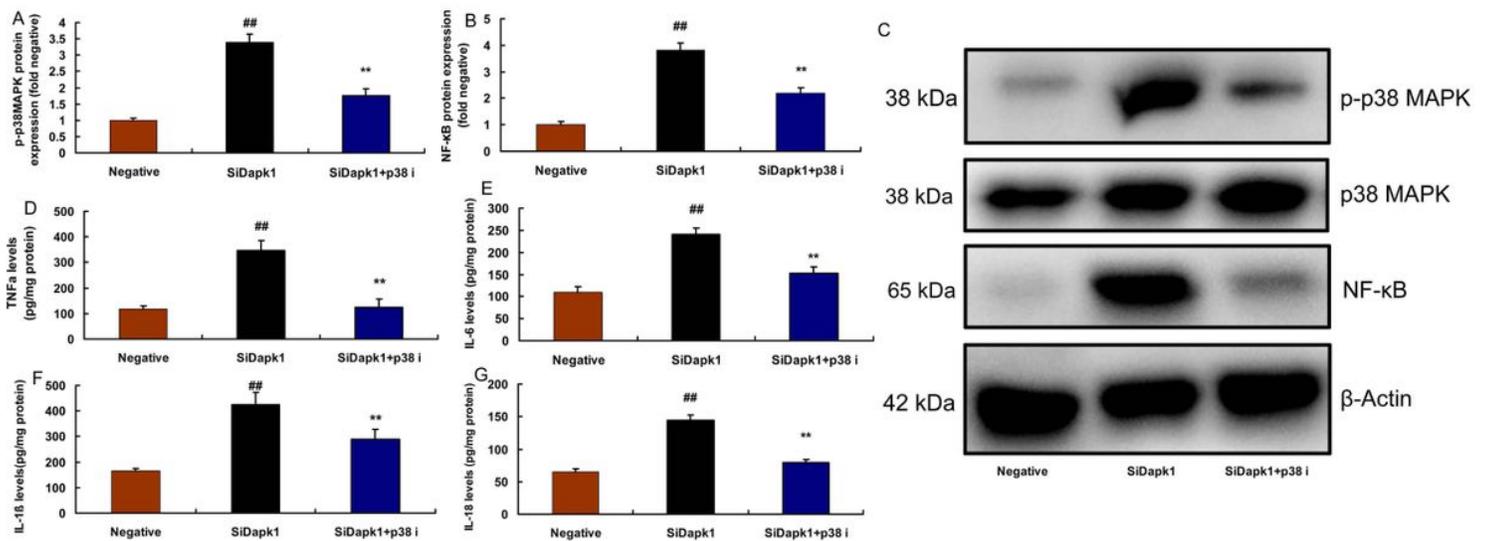
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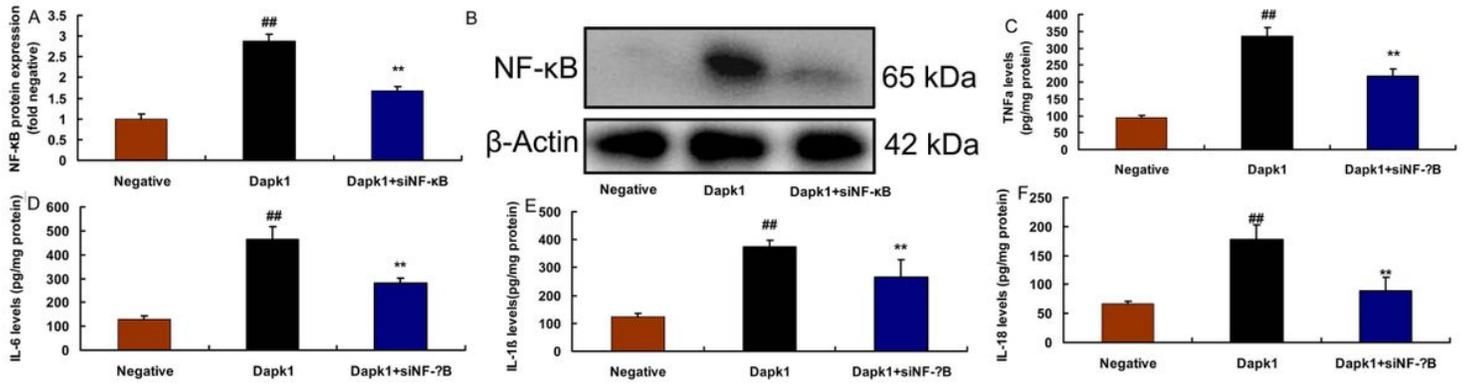
**Figure 5**

The inhibition of p38MAPK participated in the effects of Dapk1 in infantile pneumonia p-p38MAPK and NF-κB protein expression (A, B and C), TNF-α (D), IL-6 (E), IL-1β (F) and IL-18 (G) levels. Negative, negative mimic group; Dapk1, over-expression of Dapk1 group; Dapk1+ p38 i, over-expression of Dapk1 and p38 inhibitor group. ## $p < 0.01$  compared with negative mimic group; \*\* $p < 0.01$  compared with over-expression of Dapk1 group.



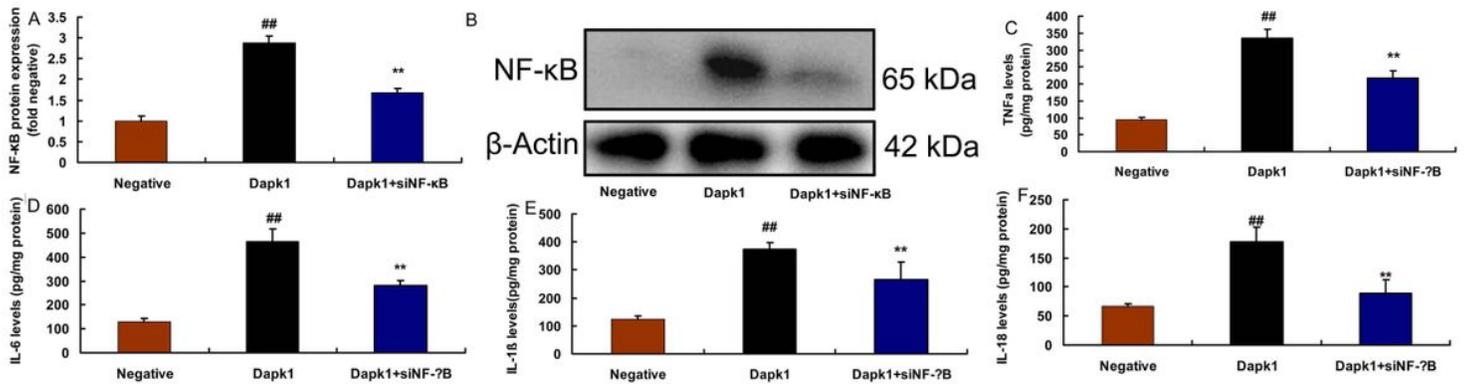
**Figure 5**

The inhibition of p38MAPK participated in the effects of Dapk1 in infantile pneumonia p-p38MAPK and NF-κB protein expression (A, B and C), TNF-α (D), IL-6 (E), IL-1β (F) and IL-18 (G) levels. Negative, negative mimic group; Dapk1, over-expression of Dapk1 group; Dapk1+ p38 i, over-expression of Dapk1 and p38 inhibitor group. ## $p < 0.01$  compared with negative mimic group; \*\* $p < 0.01$  compared with over-expression of Dapk1 group.



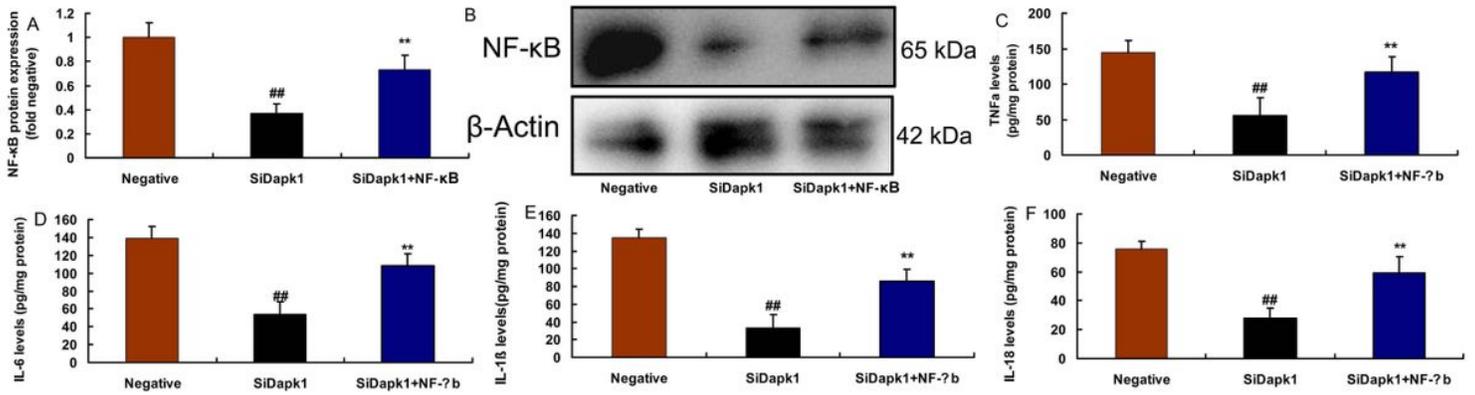
**Figure 6**

The inhibition of NF-κB participated in the effects of Dapk1 in infantile pneumonia NF-κB protein expression (A and B), TNF-α (C), IL-6 (D), IL-1β (E) and IL-18 (F) levels. Negative, negative mimic group; Dapk1, over-expression of Dapk1 group; Dapk1+ siNF-κB, over-expression of Dapk1 and down-regulation of NF-κB group. <sup>##</sup>p<0.01 compared with negative mimic group; <sup>\*\*</sup>p<0.01 compared with over-expression of Dapk1 group.



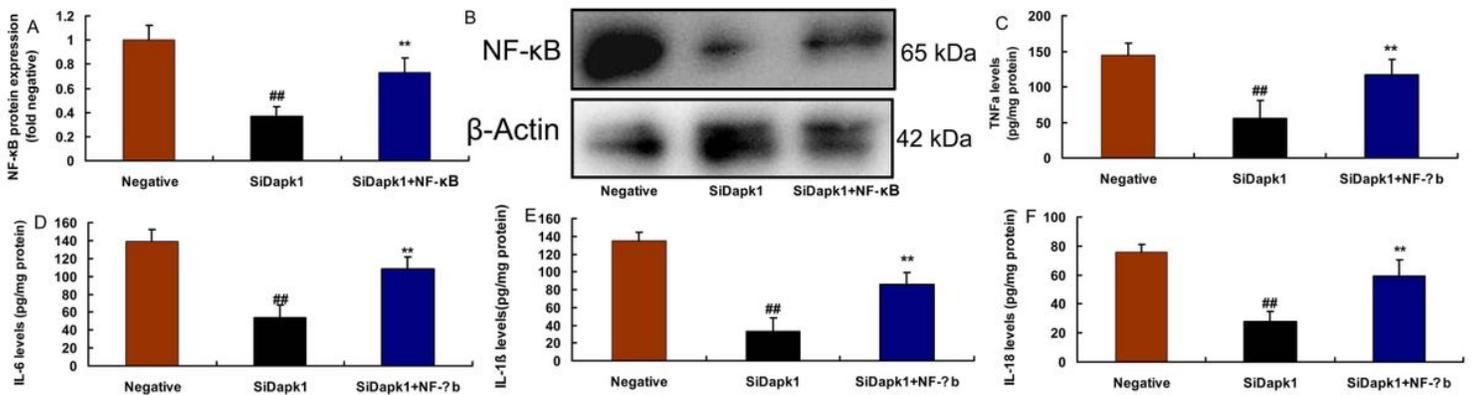
**Figure 6**

The inhibition of NF-κB participated in the effects of Dapk1 in infantile pneumonia NF-κB protein expression (A and B), TNF-α (C), IL-6 (D), IL-1β (E) and IL-18 (F) levels. Negative, negative mimic group; Dapk1, over-expression of Dapk1 group; Dapk1+ siNF-κB, over-expression of Dapk1 and down-regulation of NF-κB group. <sup>##</sup>p<0.01 compared with negative mimic group; <sup>\*\*</sup>p<0.01 compared with over-expression of Dapk1 group.



**Figure 7**

The induction of NF-κB participated in the effects of Dapk1 in infantile pneumonia NF-κB protein expression (A and B), TNF-α (C), IL-6 (D), IL-1β (E) and IL-18 (F) levels. Negative, negative mimic group; siDapk1, down-regulation of Dapk1 group; siDapk1+NF-κB, down-regulation of Dapk1 and over-expression of NF-κB group. ##p<0.01 compared with negative mimic group; \*\*p<0.01 compared with down-regulation of Dapk1 group.



**Figure 7**

The induction of NF-κB participated in the effects of Dapk1 in infantile pneumonia NF-κB protein expression (A and B), TNF-α (C), IL-6 (D), IL-1β (E) and IL-18 (F) levels. Negative, negative mimic group; siDapk1, down-regulation of Dapk1 group; siDapk1+NF-κB, down-regulation of Dapk1 and over-expression of NF-κB group. ##p<0.01 compared with negative mimic group; \*\*p<0.01 compared with down-regulation of Dapk1 group.