

Inhibitor κ B α protein therapy alleviates severe pneumonia through inhibition of nuclear factor κ B

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Abstract. To investigate the effect of inhibitor κ B α (I κ B α) on severe pneumonia and explain the mechanisms of nuclear factor κ B (NF- κ B), the activation of NF- κ B was induced in Sprague-Dawley (SD) rats infected with *Klebsiella pneumoniae* (*K. pneumoniae*). The rats were then treated with differing concentrations of I κ B α protein. A histological analysis was performed to compare the lung structure prior to and following treatment, and an immunohistochemistry assay was used to detect NF- κ B activity. In addition, the expression of certain inflammatory factors was detected using a protein chip assay. The severe pneumonia rat model was successfully produced and in model rats, NF- κ B was activated by *K. pneumoniae*. Following treatment with I κ B α , the activity of NF- κ B was inhibited and pneumonia symptoms in model rats were alleviated. Furthermore, the expression of a number of inflammatory factors including tumor necrosis factor α (TNF- α), interleukin 6 (IL-6), interferon γ (IFN- γ) and monocyte chemoattractant protein-1 (MCP-1) were also inhibited. The current study demonstrates that NF- κ B inhibition with I κ B α protein therapy prevents the development of pneumonia in a *K. pneumoniae* rat model. The therapeutic effect is indicated by the responses of proinflammatory factors, including TNF- α , IL-6, IFN- γ and MCP-1.

Introduction

Severe pneumonia is an acute respiratory infection that leads to a large number of mortalities worldwide (1). *Klebsiella pneumoniae* (*K. pneumoniae*) is a common gram-negative bacterium that can cause destructive infections in human lungs through inflammation and hemorrhage. Lung infections

caused by *K. pneumoniae* are typically necrotic (2,3). Pneumonia caused by *K. pneumoniae* has a high mortality rate and in alcoholic patients, it is almost 100% (4).

At present, antibiotic therapy is the standard treatment for pneumonia caused by *K. pneumoniae*; however, a number of *K. pneumoniae* strains are resistant to antibiotics, including ciprofloxacin (5), carbapenem (6) and colistin (7). Due to the ineffectiveness of the antibiotics currently used, novel therapies that *K. pneumoniae* is not resistant to should be developed.

Nuclear factor κ B (NF- κ B) is an important transcription factor in chronic inflammatory diseases (8) and can regulate numerous inflammatory responses. It is activated by certain inflammatory factors including interleukin 1 β (IL-1 β), tumor necrosis factor α (TNF- α) and bacterial lipopolysaccharides (LPS) (9-11). In unstimulated cells, the activity is inhibited by the inhibitor protein κ B (I κ B), of which I κ B α has been well studied (12). When cells are stimulated, NF- κ B related signaling pathways are activated by signals including reactive oxygen species (ROS), IL-1 and TNF- α (13). By contrast, I κ B is phosphorylated and ubiquitinated by I κ B kinases, and eventually degraded by proteasomes (14). Developing NF- κ B targeted therapy to treat severe pneumonia has been a focus of previous research (15,16). However, to the best of our knowledge, the effectiveness of I κ B α treatment for severe pneumonia has not yet been investigated.

In the present study, a pneumonia model in rats was produced by infecting rats with *K. pneumoniae*. These rats were subsequently treated with I κ B α protein. The study aimed to provide an insight into the development of a novel therapy for severe pneumonia and improve understanding of the mechanisms of I κ B α and NF- κ B.

Materials and methods

Rat model. The *K. pneumoniae* standard strain (ATCC700603) was provided by the National Center for Medical Culture Collections (Beijing, China). *K. pneumoniae* was subcultured onto blood agar containing 5% blood (Shanghai Yeasen Biotechnology Co., Ltd., Shanghai, China) at 37°C for 18-24 h. The inoculum was subsequently diluted to 10 colony forming units/ml (CFU/ml) by ddH₂O. A total of 40 Sprague-Dawley (SD) male rats (Shanghai Laboratory Animal Center, Shanghai, China) weighing 180-260 g and aged 5-8 months were used. They were housed in stainless steel wire mesh cages in an animal room that was maintained at 22±2°C and

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40-70% relative humidity with air ventilation 10-15 times/h and natural lighting. Normal feeding and water were provided. A rat model of pneumonia was established as previously described (17). Briefly, the SD rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (Shanghai Xinfan Biological Technology Co., Ltd., Shanghai, China). The trachea of each rat was instilled with 0.3 ml prepared inoculum. Then, the inoculated rats were held upright for 20 sec. The clinical signs of the inoculated rats including symptoms of dysphoria, activity situation, body temperature, breath, hair, somnolence, appetite, response and weight were observed every day. Age- and gender-matched rats inoculated with 0.3 ml sterile physiological saline solution were used as controls. After 7 days, the randomly selected model rats and the control rats were sacrificed by placing the rats in a chamber containing CO₂. Arterial blood was taken and analyzed by a blood-gas analyzer (NOVA Biomedical, Waltham, MA, USA). Lung lobe obtained by resection was dried at 80°C for 20 h and the ratio of the fresh weight to dry weight (F/D) was calculated. The left principal bronchus was ligated and the alveoli of the right lungs were lavaged with a bronchoscope. The numbers of white blood cells (WBCs) and neutrophils (PMNs) in the bronchoalveolar lavage fluid were assessed. The present study was approved by the ethics committee of Changhai Hospital (Shanghai, China).

I κ B α treatment. The model rats were divided into four groups with 10 rats in each. The four groups of rats were injected with 0.2 ml physiological saline (PS; control), or 10, 20 or 40 mg/kg I κ B α protein (Abcam, Cambridge, UK) respectively. After 15 consecutive days, the animals were sacrificed by placing the rats in a chamber containing CO₂. The F/D of superior lobe of right lung was analyzed. The lavage fluid was collected and the numbers of WBCs and PMNs were checked using a blood cell counter (Beckman Coulter, Inc., Brea, CA, USA). Blood-gas analysis of arterial blood using a blood gas analyzer (Nova Biomedical) was also performed.

Histological analysis. The lung tissues of rats were fixed with 10% neutral formalin solution at room temperature for 24 h. Then, the lungs were dehydrated with increasing concentrations of ethanol, infiltrated with xylene and embedded in paraffin. The lungs were sectioned to 5-8 μ m and stained with hematoxylin and eosin (H&E). The sections were then observed with a microscope.

Immunohistochemistry (IHC) assay. Sections with a thickness of 4 μ m were mounted onto slides that were coated with adhesive. Slides were deparaffinized with xylene, rehydrated with graded concentrations of ethanol, and incubated with H₂O₂ at 37°C for 10 min. Then, the sections were washed with phosphate-buffered saline (PBS) and heat-mediated antigen retrieval was performed using a microwave. Following blocking using 5% (v/v) normal goat serum (Shanghai Yeasen Biotechnology Co., Ltd.) at 37°C for 10 min, sections were incubated overnight with NF- κ B p105/p50 monoclonal antibody (ab32360; Abcam) at a dilution of 1:1,000. Washing with PBS was completed three times and the sections were incubated with biotin-conjugated goat-anti-rabbit immunoglobulin G secondary antibody (diluted with 3% bovine serum

albumin/PBS; ab64257; Abcam) at 37°C for 30 min. Further washing with PBS of the sections was completed three times, prior to incubation with horseradish peroxidase-conjugated streptavidin at 37°C for 30 min. The sections were then washed again using PBS three times and 3,3'-diaminobenzidine (DAB) was used as chromogenic agent. The streptavidin-peroxidase IHC kit was purchased from Maxim Biotech, Inc. (Rockville, MD, USA).

Protein chip detection. A protein chip assay kit (QAR-INF-1; Raybiotech, Inc., Norcross, GA, USA) was used to detect the expression levels of inflammatory factors in rats with severe pneumonia. The assay was performed according to the manufacturer's instructions. In brief, the chips were dried at room temperature for 2 h and blocked using block buffer for 1 h. Then, the chips were incubated with serum for 2 h. Following washing, chips were incubated with antibody (a biotin-labeled antibody mixture diluted with blocking buffer, both from the QAR-INF-1 kit) for 2 h and with cyanine 3 (CY3) fluorochrome for another 3 h. The fluorescence intensity was detected using the GenePix 4000B scanner (Molecular Devices, LLC, Sunnyvale, CA, USA).

Statistical analysis. The results were presented as mean \pm standard deviation (SD). All statistical analyses were performed using SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA). Comparisons between groups were conducted using Student's t-test, and P<0.05 was considered to indicate a statistically significant difference.

Results

Animal model. Following inoculation, the model rats appeared ill and exhibited symptoms of dysphoria, fever, polypnea, somnolence, decreased appetite, slow response and weight loss. These symptoms increased in intensity over time. However, no significant changes were observed in control rats. Compared with the control rats, the oxyhemoglobin saturation (SaO₂) and arterial partial pressure of oxygen (PaO₂) of model rats decreased significantly (P<0.05; Table I). In addition, partial pressure of carbon dioxide (PaCO₂), carbon dioxide (CO₂), WBC, PMNs and F/D of model rats increased significantly (P<0.05; Table I). The results indicate that the production of a severe pneumonia rat model was successful.

Activity of NF- κ B. As presented in Fig. 1, the IHC results demonstrate that in model rats, the cytoplasm and cell nuclei of NF- κ B positive cells were colored brown. Following middle (20 mg/kg) and high dose (40 mg/kg) I κ B α protein treatment, this brown coloration was significantly reduced.

Change in the indices of pneumonia. Table II compares the level of indices associated with pneumonia in rats treated with PS or I κ B α . The SaO₂ and PaO₂ of model rats in the middle and high dose I κ B α treatment groups increased significantly compared with their values in rats treated with PS (P<0.05), while significant reductions in PaCO₂, CO₂, the number of WBC and PMNs and F/D were observed in rats treated with \geq 20 mg/kg I κ B α (P<0.05; Table II).

Table I. Changes in incidence of pneumonia in model rats and control rats.

Group	SaO ₂ (%)	PaO ₂ (kPa)	PaCO ₂ (kPa)	CO ₂ (ml/dl)	WBC (10 ⁹ /l)	PMN (10 ⁹ /l)	F/D
Control	98.47±4.23	12.43±1.12	6.56±0.32	18.87±0.47	0.45±0.02	0.04±0.00	3.42±0.23
Model	72.36±5.38 ^a	8.03±0.42 ^a	9.25±0.58 ^a	30.21±0.62 ^a	2.48±0.15 ^a	2.42±0.25 ^a	5.46±0.42 ^a

Mean ± standard deviation (n=10). ^aP<0.05 compared with the control group. SaO₂, oxyhemoglobin saturation; PaO₂, arterial partial pressure of oxygen; PaCO₂, partial pressure of carbon dioxide; CO₂, carbon dioxide; WBC, white blood cell; PMN, neutrophil; F/D, ratio of fresh weight to dry weight.

Table II. Changes in indices of pneumonia in model rats following different treatments.

Group	SaO ₂ (%)	PaO ₂ (kPa)	PaCO ₂ (kPa)	CO ₂ (ml/dl)	WBC (10 ⁹ /l)	PMN (10 ⁹ /l)	F/D
Control	100.25±3.56	13.58±0.82	5.87±0.42	17.56±1.08	0.45±0.02	0.041±0.002	3.12±0.12
Physiological saline	70.48±2.24	7.93±0.35	10.55±0.26	32.23±1.78	2.48±0.15	2.245±0.184	6.12±0.23
10 mg/kg IκBα	72.89±2.56	8.25±0.46	9.23±1.58	30.68±1.21	2.03±0.08	2.034±0.076	5.87±0.21
20 mg/kg IκBα	92.46±4.21 ^a	12.54±1.12 ^a	6.86±0.85 ^a	19.34±2.05 ^a	1.21±0.01 ^a	0.123±0.014 ^a	3.85±0.24 ^a
40 mg/kg IκBα	96.87±3.54 ^a	12.26±0.87 ^a	6.21±0.35 ^a	18.58±1.02 ^a	0.87±0.03 ^a	0.089±0.003 ^a	3.22±0.38 ^a

^aP<0.05 compared with the physiological saline treatment group. Mean ± standard deviation (n=10). SaO₂, oxyhemoglobin saturation; PaO₂, arterial partial pressure of oxygen; PaCO₂, partial pressure of carbon dioxide; CO₂, carbon dioxide; WBC, white blood cell; PMN, neutrophils; F/D, ratio of fresh weight to dry weight; IκBα, inhibitor κBα.

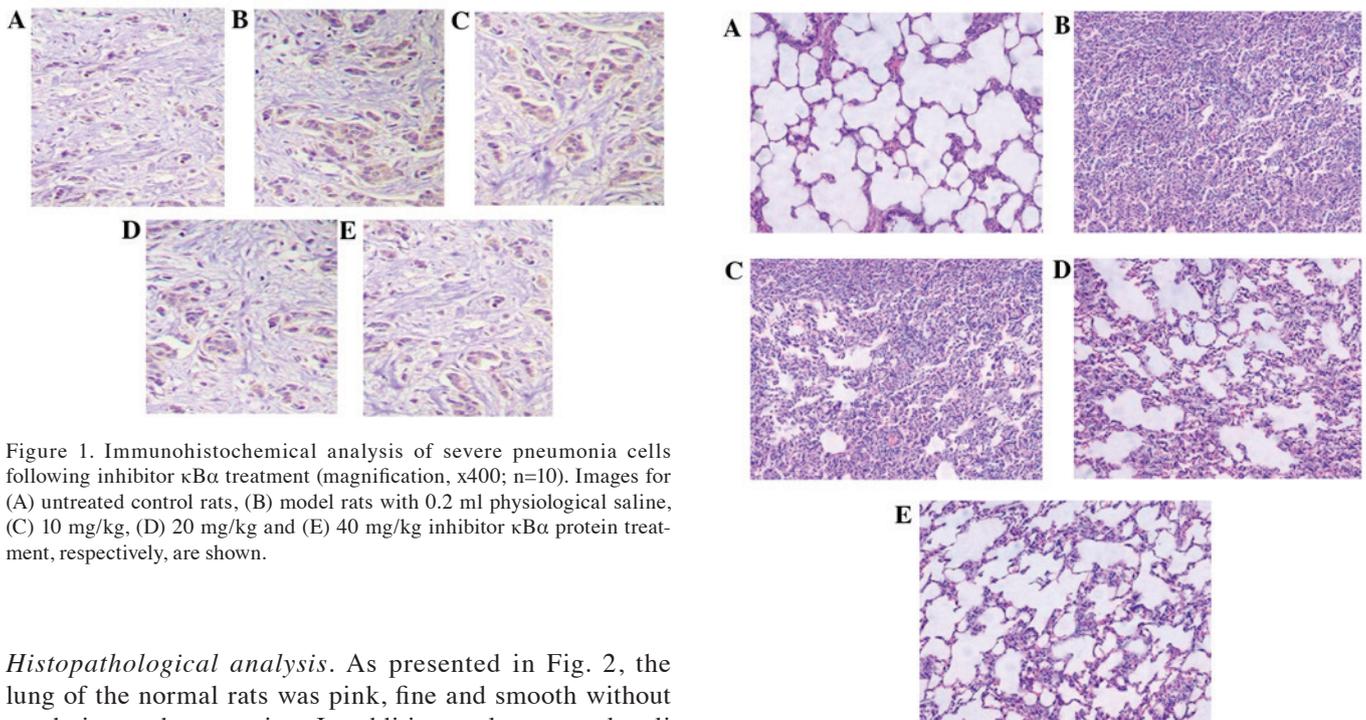


Figure 1. Immunohistochemical analysis of severe pneumonia cells following inhibitor κBα treatment (magnification, x400; n=10). Images for (A) untreated control rats, (B) model rats with 0.2 ml physiological saline, (C) 10 mg/kg, (D) 20 mg/kg and (E) 40 mg/kg inhibitor κBα protein treatment, respectively, are shown.

Histopathological analysis. As presented in Fig. 2, the lung of the normal rats was pink, fine and smooth without exudation and congestion. In addition, pulmonary alveoli exhibited clear structures, thin walls and clean alveolar spaces. However, the lung of the pneumonia model rats was dark red with exudation of blood and purulence. Diffuse pulmonary consolidation was observed. Following treatment with IκBα, exudation of blood and purulence of lung in model rats decreased and the group receiving high dose IκBα treatment demonstrated the least exudation. IκBα treatment also alleviated the damage of pulmonary alveolus structure in model rats (Fig. 2).

Figure 2. Histopathological analysis of the lung in different rat groups (magnification, x100; n=10). Images for (A) Untreated control rats, (B) model rats receiving 0.2 ml physiological saline, (C) 10 mg/kg, (D) 20 mg/kg and (E) 40 mg/kg inhibitor κBα protein treatment, respectively, are shown.

Expression of inflammatory factors. As presented in Fig. 3, the expression levels of interleukin 6 (IL-6), TNF-α, interferon γ (IFN-γ) and monocyte chemoattractant protein-1 (MCP-1) in PS

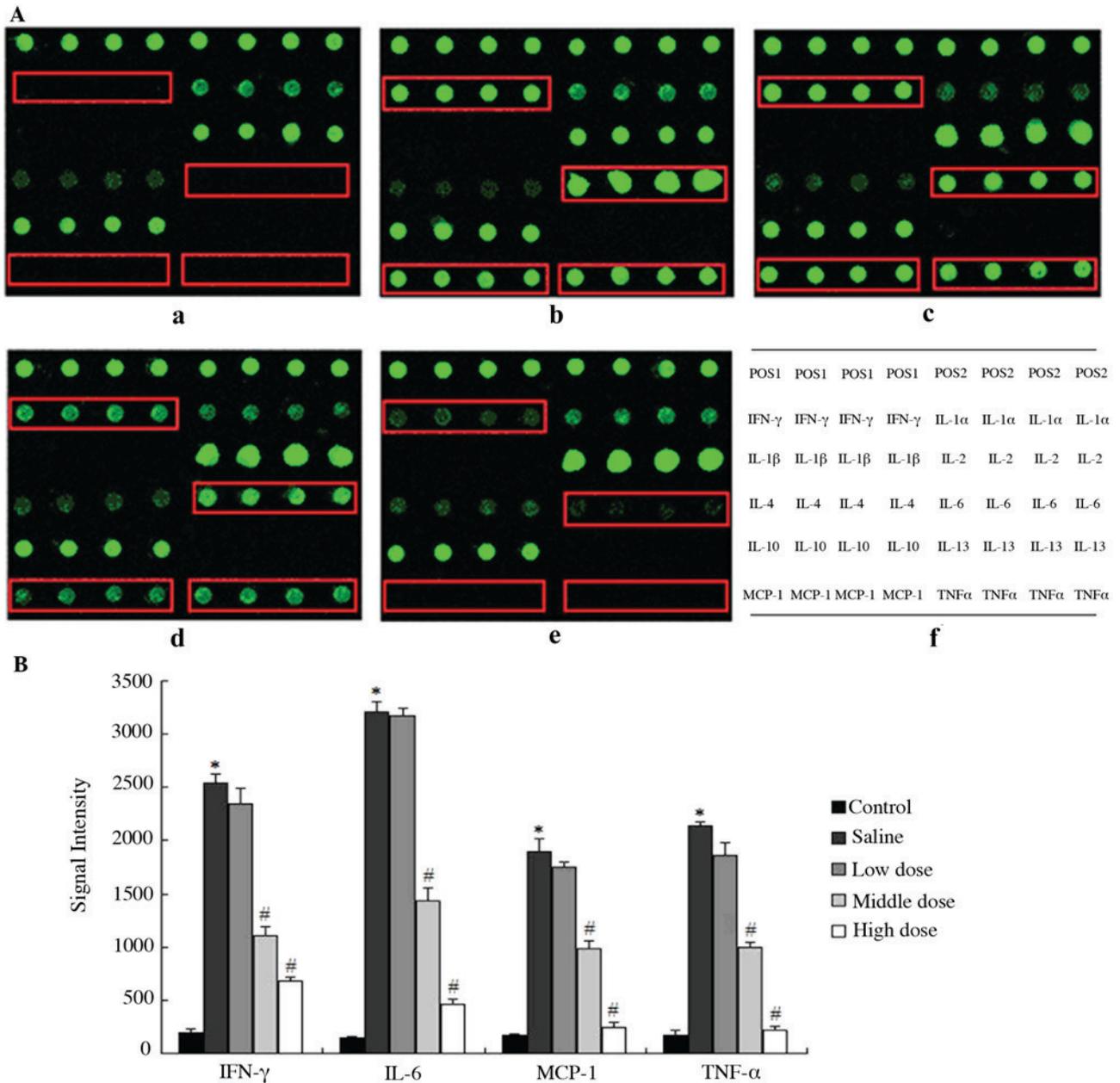


Figure 3. Expression of inflammatory factors. (A) Results of protein chip assay for (a) untreated control rats, (b) model rats with 0.2 ml physiological saline, (c) 10 mg/kg, (d) 20 mg/kg and (e) 40 mg/kg IκBα protein treatment respectively; (f) the distribution of the inflammatory factors in each plate. (B) Relative expression of TNF-α, IL-6, IFN-γ and MCP-1. *P<0.05 vs. control; #P<0.05 vs. the physiological saline-treated group. POS, the quality control index of the chip; IκBα, inhibitor κBα; TNF-α, tumor necrosis factor α; IL, interleukin; IFN-γ, interferon γ; MCP-1, monocyte chemoattractant protein-1.

rats were significantly higher than the control group (P<0.05). Following treatment with middle and high dose IκBα, levels of IL-6, TNF-α, IFN-γ and MCP-1 expression were significantly lower than those indicated for the PS group (P<0.05, Fig. 3B).

Discussion

In the current study, a rat model for severe pneumonia was produced by infecting SD rats with *K. pneumoniae*. In the model rats, the activity of NF-κB was significantly higher than that of controls, suggesting that NF-κB was activated and this activation may facilitate inflammation. By contrast, when the model rats were treated with IκBα, the activity of NF-κB was inhibited, and there was a reduction in PaCO₂, CO₂, F/D and

the numbers of WBC and PMNs. The expression of inflammatory factors IL-6, TNF-α, IFN-γ and MCP-1 also decreased. Thus, expression levels of inflammatory factors may be indicators of NF-κB activation.

One well-accepted hypothesis about NF-κB is that NF-κB is a response to the prototypical proinflammatory cytokines TNFα and IL-1, which contribute to host defenses against a number of pulmonary bacteria (18,19). However, the association between NF-κB and inflammation cannot be interpreted clearly, as no responses of IL-1α and IL-1β were found in the current study. Jones *et al* (20) have suggested that the requirement of TNF-α and IL-1 receptors for NF-κB activation in pneumonia resulting from infection with *Streptococcus pneumoniae* was caused by more than gram-negative

stimuli (*Escherichia coli*). There is a discrepancy in the current study, as there are indications that TNF- α may be necessary for NF- κ B activation in *K. pneumoniae*, however this may be due to the different responses of IL-1 by different gram-negative bacteria.

Another explanation is that activation of NF- κ B may be related to macrophage activation through certain cytokines (IL-6 and IFN- γ) and chemokines such as MCP-1. IFN- γ can contribute to the activation and differentiation of macrophages and subsequent induction of the inflammatory response (21). Chemokine MCP-1 may be a factor that acts on macrophages and monocytes and contributes to the recruitment of PMNs (22). In the current study, similar to TNF- α , expression of IL-6, IFN- γ and MCP-1 were induced when NF- κ B was activated by bacterial infection. Then, following inhibition of NF- κ B by I κ B α , the expression of IL-6, IFN- γ and MCP-1 decreased. PMNs and WBC in pulmonary alveoli demonstrated a similar trend. These results suggest that the expression of proinflammatory cytokines and chemokines, and an alveolar PMN response are important in the resolution of bacteria infected pneumonia.

In the present study, it is worth noting that no significant difference was observed in the expression of IL-2 between control and PS treated rats, but IL-2 expression was induced following treatment with I κ B α protein. Leung and Nabel (23) have reported that the human T lymphotropic virus-I may induce expression of the IL-2 receptor by a NF- κ B-like factor. It is also indicated that a similar site to κ B exists upstream of IL-2 receptor α . However, the results of the current study demonstrated that the expression of IL-2 appears to be dependent on I κ B α rather than NF- κ B. The mechanisms of NF- κ B on inflammatory factors are complex and remain unclear. Further studies are needed to evaluate the NF- κ B pathway.

In conclusion, the current study demonstrates that NF- κ B inhibition with I κ B α protein therapy prevents the development of pneumonia caused by *K. pneumoniae* in a rat model. The therapeutic effect may occur through the responses of several proinflammatory factors including TNF- α , IL-6, IFN- γ and MCP-1. However, to clarify the mechanisms of NF- κ B and inflammation, further studies focused on pneumonia infected by other bacteria in other animal models should be performed.

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