

# The role of ICOSL in children with neutrophilic asthma

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## Research

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# Abstract

**Background:** It has been shown that certain severe and refractory asthma cases are due to neutrophil and not eosinophil infiltration. ICOSL (Inducible costimulatory molecular ligand) expression is closely associated with tumor and autoimmune diseases, while a limited amount of data has been published regarding the significance of ICOSL in children with neutrophilic asthma. The present study aimed to explore the abnormal expression of ICOSL in peripheral blood and bronchoalveolar lavage fluid (BALF) samples of children with neutrophilic asthma and their clinical significance.

**Methods:** The present clinical study selected children who met the diagnostic criteria of asthma from the children's Hospital of Suchow University and excluded the patients with positive etiology. The children who were admitted to the hospital for foreign body inhalation in the same period were collected as the control group. The children with more than 50% (the percentage of neutrophils in BALF was 95% of the percentile in the control group) of neutrophils in BALF samples were assigned to the neutrophilic asthma group (NA group), whereas the remaining subjects comprised the asthma group (A group). The expression levels of ICOSL, IL-4, IL-17, IFN- $\gamma$ , neutrophil elastase (NE) and matrix metalloproteinase (MMP)-9 were detected in plasma and BALF samples by enzyme-linked immunosorbent assays in order to analyze the differences in the levels of cytokines and clinical characteristics between children with neutrophilic asthma and non-neutrophilic asthma. Moreover, the potential mechanism of ICOSL in neutrophilic asthma was explored.

**Results:** In strict accordance with the diagnostic criteria of asthma and following exclusion of pathogenic positive children, 32 children were finally enrolled, including 12 children in the NA group and 20 children in the A group. The mean hospitalization time of the NA group was longer than that of the A group ( $P < 0.05$ ). The concentration levels of ICOSL, IL-17, NE and MMP9 of the NA group in plasma and BALF samples were higher than those in the A group, while the levels of IFN- $\gamma$  exhibited the opposite trend. A significant correlation was evident between ICOSL and IL-17 levels in plasma ( $r = 0.753$ ,  $P = 0.012$ ) and BALF ( $r = 0.774$ ,  $P = 0.009$ ) samples of the NA group.

**Conclusion:** Children with asthma exhibited an immunity imbalance of Th1/Th2/Th17, whereas neutrophilic asthma children were more severely affected. The clinical treatment was considerably difficult and the hospitalization time was longer. ICOSL may regulate the secretion of IL-17 by Th17 and increase the levels of NE and MMP9, which are involved in the development of immune inflammation in neutrophils.

## Background

Bronchial asthma has been highly valued by previous studies due to its recurrent state, its effects on children's growth and development and due to the reduction in the quality of life. Bronchial asthma is also considered a severe financial burden. Approximately 30 million asthmatic patients exist in China and among them, children with asthma account for almost 1/3 of the total number of cases<sup>[11]</sup>. The

pathogenesis of asthma is complex. Th1/Th2 imbalance, which is considered the dominant factor in Th2 imbalance, is the most important immunological mechanism involved in the pathogenesis of eosinophilic asthma. However, this view is not sufficient to explain the development of all types of asthma<sup>[1,2]</sup>.

Bronchial asthma is the most common noninfectious disease in children. Approximately, 300 million asthmatic patients exist in the world. The prevalence of this disease has increased significantly in middle and low-income countries during the last years<sup>[1-2]</sup>. It is believed that the increase of eosinophils is one of the characteristics of airway inflammation in asthma. However, in recent years, certain patients did not exhibit increased sputum eosinophil number and more than 50% of asthmatic subjects presented with neutrophil infiltration of airway inflammation<sup>[3-4]</sup>. The number of neutrophils was also elevated during exacerbations and increased duration of asthma<sup>[3-4]</sup>. The role of neutrophil infiltration in the treatment of severe asthma, fatal asthma, chronic persistent asthma and refractory asthma is increasingly emphasized<sup>[5-7]</sup>. However, the main pathogenesis of neutrophil infiltration in asthma is not fully understood. It is of particular significance to study and understand the role of neutrophil infiltration in the pathogenesis of asthma.

ICOSL (inducible co-stimulator ligand), also known as B7-H2, B7h, GL50, or B7RP-1, is an important member of the ICOS/ICOSL signaling pathway together with its receptor ICOS. It has been shown that the ICOS/ICOSL signaling pathway is associated with the inflammatory response, tumor and transplant rejection and the development of autoimmune diseases<sup>[8]</sup>. However, research on asthma is rare. In the present study, we investigated the expression levels of ICOSL in peripheral blood and bronchoalveolar lavage fluid (bronchoalveolar lavage fluid, BALF) samples from children with neutrophilic asthma and their correlation with Th1, Th2 and Th17 secretion of cytokines. The clinical significance of the expression of ICOSL in peripheral blood and BALF samples of children with neutrophilic asthma and the association between the concentration levels of cytokines were further discussed.

## Methods

### Study Design

Collection of peripheral blood and BALF

The samples were collected from the children with asthma within 24 h following admission and from the children that required removal of foreign bodies prior to surgery. A total of 2 ml of sample was collected from peripheral blood and BALF of children who required electronic bronchoscopy. The samples were stored at -80°C following centrifugation and used for the detection of cytokines.

Clinical data collection

The allergen test in children with asthma was performed to assess allergies. The clinical data of the children with asthma and of the control group were collected at the same time.

Cytokine detection

The samples of BALF and plasma were obtained for cytokine and ICOSL detection from the children at the time of admission and at discharge. The samples were immediately centrifuged and preserved at -80°C for subsequent assays. All the cytokines were detected using the ELISA technique. The levels of ICOSL, IFN- $\gamma$ , IL-4, IL-17, MMP9 and NE were assessed with ELISA kits (R&D systems, America).

#### Allergen detection

The allergic condition of the children was investigated using blood allergen or skin allergen origin prick tests.

## Participants

Children who were admitted to the Respiratory Medicine of Children's Hospital of Soochow University with the diagnostic criteria of asthma<sup>[9]</sup> were selected from August 2014 to July 2016. The following exclusion criteria were used: Positive detection of BALF pathogen, which included respiratory syncytial virus, adenovirus, influenza virus A, B, parainfluenza virus 1, 2, 3, Boka virus, human metapneumovirus, rhinovirus, mycoplasma, chlamydia pneumonia, tuberculosis and bacteriological detection.

A total of 32 asthmatic children were recruited and were classified according to the BALF cell classification with more than 50% neutrophils. The percentage of neutrophils in BALF was the 95th percentile in the control group. The remaining groups were the neutrophilic asthma group (NA group) and the asthma group (A group). Concomitantly, the children that were examined with the electronic bronchoscope for the presence of foreign bodies in the Children's Hospital of the Soochow University comprised the control group (C group). A total of 16 cases were selected (11 males and 5 females). The C group did not present with allergic diseases and exhibited no previous history of this type of disease. The C group included subjects that were nearly 4 weeks without the use of any drugs and did not exhibit a previous history of infection.

The present study was approved by the ethics committee of the Children's Hospital of Soochow University. The guardians of the children provided signed informed consent and all the children with asthma were treated with conventional treatment<sup>[10]</sup>. According to the condition of the children, they were administered with inhaled corticosteroids or oral corticosteroids, oxygen, suitable fluid support and other comprehensive support.

The present study was performed according to the protocol approved by the Ethics committee of the Children's Hospital of Soochow University.

## Statistical analysis

The data were analyzed using the PASW 18.0 statistical package and the measurement data were expressed as mean  $\pm$  SD. The count data were expressed as percentages or rates. The rank sum test was

used for comparison between the groups and the consistency analysis was performed by the kappa consistency test. The differences were significant when  $P < 0.05$ .

## Result

### General Information

A total of 32 children with asthma were enrolled in the present study. All children with asthma were admitted to the hospital with cough and wheezing symptoms, including 2 cases with oxygen inhalation prior to or following admission in the NA group. A total of 3 cases obtained from outpatient or admission were treated by methylprednisolone and hydrocortisone sodium succinate intravenous administration. A total of 12 children inhaled pulmicort respules more than 3 times, whereas one case was treated with oxygen inhalation prior to or following admission in group A. A total of 6 cases from outpatient visits or admission were treated by methylprednisolone and hydrocortisone sodium succinate intravenously. A total of 12 children inhaled pulmicort respules more than 3 times. The length of hospital stay in the NA group was the longest and the hospitalization time ranged between 6 and 21 days. The hospitalization time of the A group was between 2 and 15 days, whereas the C group exhibited the shortest, hospitalization time ranging between 4 and 10 days. The differences were significant ( $P < 0.05$ , Table 1).

Table 1  
Comparison of clinical basic information

	NA group(12)	A group(20)	C group(16)	P
Gender (M/F)	8/4	16/4	11/5	0.999
Age (years)	3.03 ± 3.46	5.01 ± 3.25	3.20 ± 1.32	0.084
Hospitalization days(day)	11.18 ± 4.29	7.55 ± 4.45 <sup>a</sup>	--	0.036
Note: A is compared with group NA, $P < 0.05$ .				

### Assessment of allergic conditions in the three groups of children

In group NA, 9 cases presented with eczema or positive allergen, 7 cases with eczema, 3 cases with dust mite allergy, 2 cases with freshwater fish allergy and 2 cases with seafood allergy. There were 15 cases of group A with eczema history or allergen positive history, 11 cases with eczema, 5 cases with dust mite allergy, 2 cases with fungal allergy, 2 cases with egg allergy, 1 case with fish allergy and 1 case with shrimp allergy. No history of eczema and positive allergens was noted in the subjects of the C group.

### Cytokine Levels In Peripheral Blood And Balf Samples

3.3.1. Comparison of the levels of cytokines in the peripheral blood of the patients: The mean ICOSL levels of the NA group were  $10.06 \pm 1.01$ , which were significantly higher than those noted in the A group ( $8.16 \pm 1.25$ ) and the C group ( $7.19 \pm 2.34$ ). The differences were significant ( $P < 0.05$ ). The concentration levels of NE, IL-17, IL-4 and MMP9 of the NA group were  $445.48 \pm 40.79$ ,  $254.15 \pm 50.50$ ,  $217.88 \pm 90.74$  and  $561.42 \pm 427.76$ , respectively which were significantly higher than those noted in the A group ( $319.39 \pm 51.44$ ,  $127.02 \pm 43.10$ ,  $180.50 \pm 81.24$  and  $258.73 \pm 114.46$ , respectively) and in the C group ( $67.54 \pm 47.18$ ,  $146.27 \pm 46.67$ ,  $97.63 \pm 68.65$  and  $309.74 \pm 311.58$ , respectively). The differences were significant ( $P < 0.05$ ). The concentration levels of NE and IL-4 in group A were significantly higher than those in group C. The concentration levels of IFN- $\gamma$  in groups NA and A were  $307.81 \pm 73.52$  and  $285.88 \pm 91.23$ , respectively which were significantly lower than those of the group C ( $397.11 \pm 72.91$ , Fig. 1).

3.3.2. Comparison of the levels of cytokines in BALF: The concentration levels of ICOSL in the NA group were  $0.98 \pm 0.09$  and were significantly higher than those of the A group ( $0.33 \pm 0.09$ ) and the C group ( $0.43 \pm 0.21$ ,  $P < 0.05$ ). The mean concentration levels of NE, IL-17 and MMP9 in the NA group were  $37.70 \pm 6.93$ ,  $18.47 \pm 2.33$  and  $58.62 \pm 11.30$ , respectively, which were significantly higher than those of the A group ( $15.87 \pm 9.17$ ,  $9.59 \pm 3.52$  and  $40.16 \pm 18.19$ , respectively,  $P < 0.05$ ) and of the C group ( $27.33 \pm 6.01$ ,  $6.69 \pm 4.09$ ,  $27.66 \pm 15.74$ , respectively,  $P < 0.05$ ). The concentration levels of MMP9 in group A were significantly higher than those in group C. The expression levels of IFN- $\gamma$  in group NA were  $489.66 \pm 88.08$ , which were lower than those in group A ( $706.37 \pm 106.33$ ) and those in group C ( $666.42 \pm 92.80$ ). The differences were significant ( $P < 0.05$ ). IL-17 levels in group NA were significantly higher than those in group C, whereas the concentration levels of IL-4 in groups NA and A were significantly higher than those in group C (Fig. 2).

3.3.3. Correlation analysis of ICOSL, IFN- $\gamma$ , NE, IL-17 and MMP9 in peripheral blood and BALF samples:

(1) Correlation analysis of ICOSL, IFN- $\gamma$ , NE, IL-17 and MMP9 levels in peripheral blood: The concentration levels of ICOSL and IL-17 in peripheral blood samples exhibited positive correlation, ( $r = 0.753, P = 0.012$ ), whereas those of ICOSL and IFN- $\gamma$ , NE, MMP9 and IL-4 were less consistent ( $r = 0.633, 0.117, 0.217, 0.233, P > 0.05$ , Table 2).

Table 2  
Correlation analysis of  
ICOSL,IFN-, NE, IL-17 and  
MMP9 in peripheral blood

Y	ICOSL	
	P	r
IFN- $\gamma$	0.067	0.633
NE	0.765	0.117
IL-17	0.012	0.753
MMP9	0.576	0.217
IL-4	0.546	0.233

(2)

Correlation analysis of ICOSL, IFN- $\gamma$ , NE, IL-17 and MMP9 levels in BALF samples: The concentration levels of ICOSL and IL-17 in BALF samples exhibited positive correlation,( $r = 0.774, P = 0.009$ ), whereas ICOSL and IFN- $\gamma$ , NE, MMP9 and IL-4 levels were less consistent, $r = 0.111 \times 0.420 \times 0.253 \times 0.096 \times P > 0.05$  as demonstrated in Table 3.

Table 3  
Correlation analysis of  
ICOSL, IFN-, NE, IL-17 and  
MMP9 in BALF

Y	ICOSL	
	P	r
IFN- $\gamma$	0.76	0.111
NE	0.227	0.420
IL-17	0.009	0.774
MMP9	0.480	0.253
IL-4	0.790	0.096

## Discussion

Previous studies have demonstrated that more than 50% of the asthmatic cases involve neutrophil infiltration. This process is also observed in patients with severe asthma, fatal asthma, infantile asthma, chronic persistent asthma and refractory asthma. The present study detected the concentration levels of ICOSL in children with neutrophilic asthma in peripheral blood and BALF samples and their correlation with the concentration levels of Th1, Th2 and Th17 type cytokines.

The results indicated that the cytokines secreted by Th2 cells, notably IL-4, in BALF and peripheral blood samples of the NA and A groups were higher than those of the C group. In BALF, the concentration levels of IFN- $\gamma$ , which represented Th1, were significantly lower in the NA group than those noted in the A and C groups. These results suggested an imbalance of Th1/Th2 in children with neutrophilic asthma and asthma. The cytokines secreted by type Th2 appeared hyperactive. The data further demonstrated apparent Th1 cell dysfunction in children with neutrophilic asthma.

The pathogenesis of asthma is complicated, with epithelial cells, fibroblasts, dendritic cells, neutrophils, eosinophils, mast cells, T lymphocytes and other cells involved in chronic airway inflammation<sup>[13]</sup>. It has been proposed that Th2-related cytokines and a series of pathological processes caused by mucous secretion, cell proliferation and eosinophil infiltration, are involved in the pathogenesis and maintenance of allergic asthma that in turn correlate positively with the severity of disease symptoms. In contrast to these observations, IFN- $\gamma$  secretion by Th1 cells can inhibit the proliferation of Th2 and plays an inhibitory role in inflammation<sup>[14]</sup>. The Th1/Th2 imbalance is characterized by relative inhibition of Th1 function and relative hyperfunction of Th2 function, which causes the increase in IgE synthesis and the production of IL-4, IL-5, IL-13 and other Th2 cytokines. These processes are considered the main pathogenetic mechanisms of asthma.

IL-4 is a pleiotropic cytokine secreted by CD4<sup>+</sup>T cell subsets, B cells and mast cells. As a characteristic cytokine secreted by Th2 cells, it exhibits potent chemical chemotactic activity to eosinophils, neutrophils and other inflammatory cells. It can significantly increase the accumulation and activation of inflammatory cells in the airways, thereby inducing exacerbations of acute asthma<sup>[15, 16]</sup>. IFN- $\gamma$  is a cytokine that has been studied recently and can inhibit the proliferation of B lymphocytes and the secretion of IgG1 and IgE in B lymphocytes. In addition, it can also inhibit the expression of low affinity receptors for IgE on B cells. IFN- $\gamma$  can block allergic reactions and relieve asthma symptoms, enhancing the immune function and exerting non-specific anti-infection activity. Moreover, it can enhance phagocytic function and inhibit the survival of plasma cells, which play a protective role in the pathogenesis of asthma<sup>[17]</sup>. In recent years, it was shown that neutrophil infiltration in bronchial biopsy and induced sputum is responsible for the severe or acute exacerbation of the condition of asthmatic patients. The products of neutrophils mainly include NE, MMPs (mainly MMP-9) and IL-17<sup>[18-21]</sup>. NE mainly degrades basement membrane elastine, which is closely associated with lung tissue damage. Increased levels of NE in neutrophils can result in severe acute lung injury and ARDS. NE further promotes neutrophil recruitment and persists in the inflammatory response at accumulation sites. Lock et al.<sup>[22]</sup> indicated that in BALF samples NE levels were significantly higher in patients with acute exacerbation of

severe asthma and in patients with severe asthmatic respiratory tract submucosal inflammation regardless of the presence of eosinophils. The concentration levels of MMP9 and NE correlated positively. In recent years, increasing attention has been paid to the association between the number of Th17 cells in asthma, notably in severe asthma and the resistance in steroid treatment of asthma. Previous studies demonstrated that the Th17/IL-17 activation could aggravate airway hyperresponsiveness and airway inflammation in asthma, whereas the degree of IL-17 elevation was closely associated with the severity of asthma.<sup>[23-25]</sup>

In the present study, IFN- $\gamma$ , NE, IL-17, MMP9 and IL-4 levels were detected in peripheral blood and BALF samples of children with neutrophilic asthma, children with asthma and control subjects. It was found that the NE, IL-17 and MMP9 concentration levels in the peripheral blood of the NA group patients were significantly higher than those noted in the A and C groups. The IFN- $\gamma$  levels in the NA group were significantly lower than those noted in the A and C groups. The concentration levels of IL-4 in the NA and A groups were significantly higher than those in the C group. The concentration levels of NE and MMP9 in the NA group were significantly higher than those in the A and C groups in the BALF samples. The concentration levels of MMP9 in the A group were higher than those in the C group. The concentration levels of IFN- $\gamma$  in the NA group were lower than those in the A and C groups. IL-17 levels in the NA group were significantly higher than those noted in the C group. The concentration levels of IL-4 in the NA and A groups were significantly higher than those of the C group. The results suggested an imbalance of Th1/Th2/Th17 immune function in the NA and A groups. This effect was more severe in the NA group. The clinical data of the NA, A and C groups were analyzed and compared. The analysis indicated that the NA group exhibited the longest hospitalization and the more severe Th1/Th2/Th17 immune imbalance. The more difficult the clinical treatment, the longer the required treatment time.

The ICOSL molecule is the main member of the CD28/B7 superfamily<sup>[26]</sup>, due to its activation in T cell polarization. ICOSL causes optimization of Th1/Th2 subsets and the same type of immunoglobulin conversion plays an important role in this process. ICOSL binds to its receptor ICOS to activate the ICOS/ICOSL signaling pathway. At present, previous studies on ICOSL have mainly focused on inflammatory diseases, autoimmune diseases and tumors. ICOSL is highly expressed in inflammatory diseases<sup>[27]</sup> and is closely associated with the severity of inflammation. However, a limited number of studies have been conducted on asthma and the conclusions are not clear. Matesic et al.<sup>[28]</sup> transplanted OVA specific T cell receptor transgenic ICOS + T cells into OVA sensitized BALB/c mice demonstrating a significantly increased number of lymphocytes, macrophages, neutrophils and eosinophils in bronchoalveolar lavage fluid. However, Akbari et al. used allergen-induced mouse asthma models to investigate the role of ICOS/ICOSL signaling pathways and highlighted that ICOS stimulated the production of regulatory T cells. However, the production of Treg cells depends on the expression of high levels of ICOSL by lung dendritic cells. Treg cells inhibit the function of antigen-specific T cells and the formation of AHR<sup>[29, 30]</sup>. The present study examined the levels of ICOSL in peripheral blood and BALF samples from children with neutrophilic asthma. Moreover, the levels of ICOSL, IFN- $\gamma$ , NE, IL-17 and MMP9 were evaluated in peripheral blood and BALF samples. The results suggested that the

concentration levels of ICOSL in peripheral blood and BALF samples exhibited a positive correlation with IL-17 levels. It was shown that ICOSL may regulate IL-17 secretion by Th17 in order to recruit neutrophils in the airways, degranulate neutrophils and increase the secretion of inflammatory mediators. These processes increase the adhesion of neutrophils on endothelial cells in the respiratory tract, which in turn is involved in the occurrence and development of asthma.

## Conclusion

In conclusion, the present study demonstrated an imbalance of Th1/Th2/Th17 in children with neutrophilic asthma and asthma. The immune imbalance was more severe in the former, which was more difficult to treat and required longer hospitalization time. The concentration levels of ICOSL in the peripheral blood and BALF samples of children with neutrophilic asthma was higher than those noted in normal subjects. ICOSL may regulate the secretion of Th17 by IL-17, increase neutrophil recruitment in the airway and NE and MMP9 levels, as well as participate in the development of immunity and inflammation in neutrophilic asthma. The results of the present study may be limited due to the small sample size. Therefore, the pathogenesis of asthma requires further exploration.

## Declarations

### *Ethics approval and consent to participate*

Ethics approval This study was performed after Ethics committee of Children`s Hospital of Soochow University approval was obtained.

Consent to participate: Informed consent was obtained from all individual participants included in the study.

### *Availability of data and materials*

Data and material are available and stored in Children`s Hospital of Soochow University.

### *Competing interests*

The authors declare that they have no competing interests.

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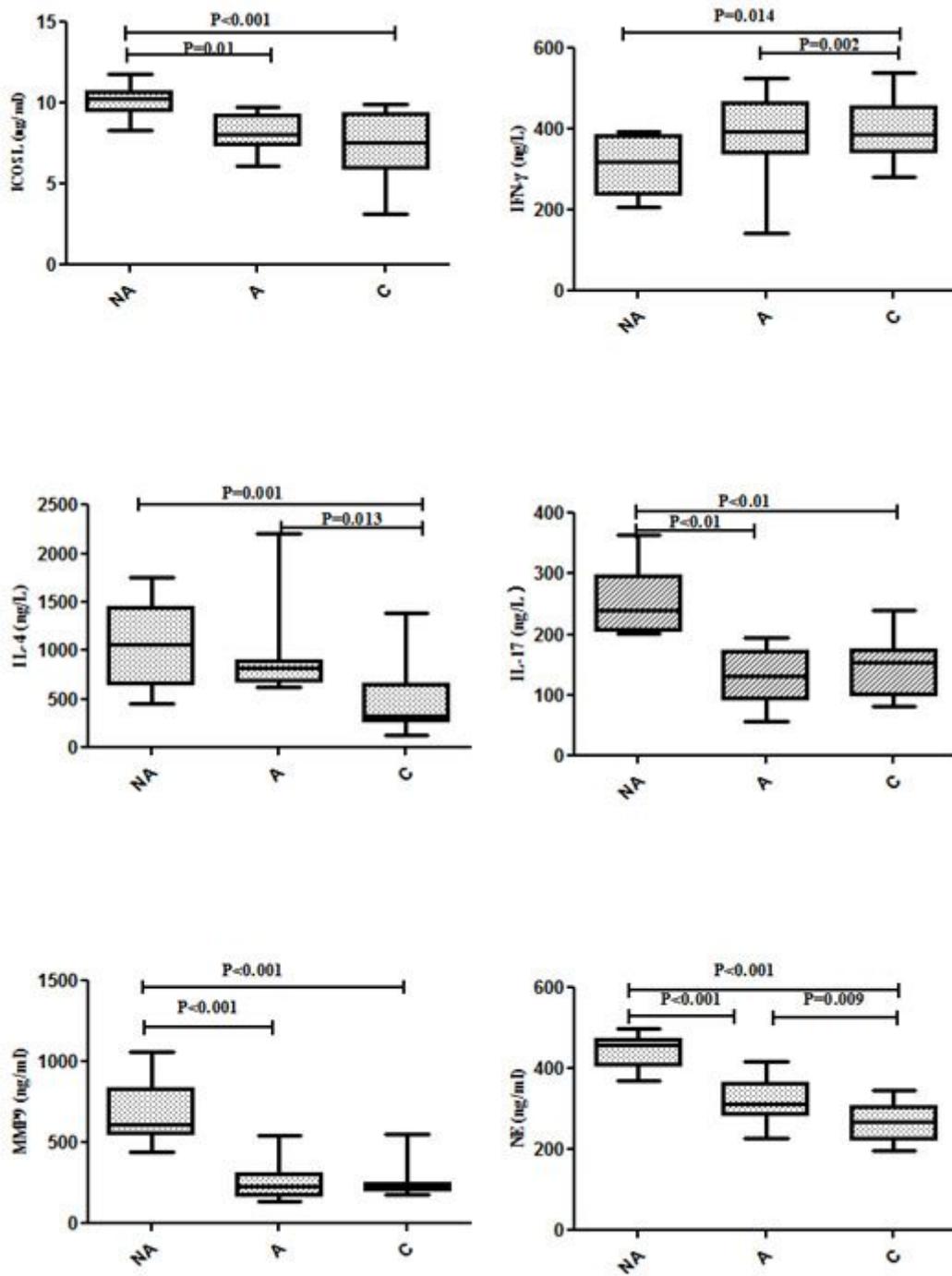
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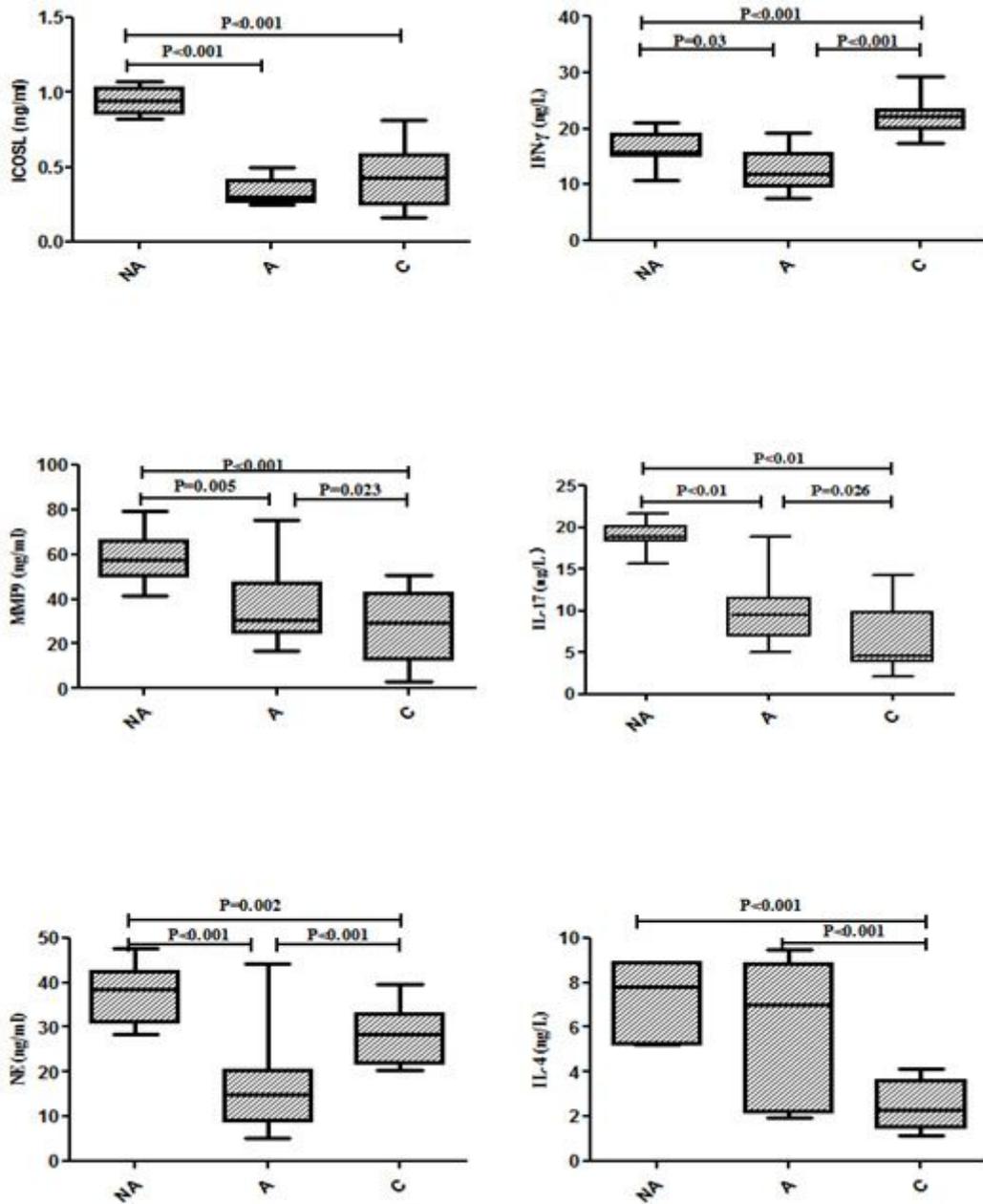
## Figures



**Figure 1**

Comparison of the levels of cytokines in peripheral blood: The mean ICOSL levels of the NA group were  $10.06 \pm 1.01$ , which were significantly higher than those noted in the A group ( $8.16 \pm 1.25$ ) and the C group ( $7.19 \pm 2.34$ ). The differences were significant ( $P < 0.05$ ). The concentration levels of NE, IL-17, IL-4 and MMP9 of the NA group were  $445.48 \pm 40.79$ ,  $254.15 \pm 50.50$ ,  $217.88 \pm 90.74$  and  $561.42 \pm 427.76$ , respectively which were significantly higher than those noted in the A group ( $319.39 \pm 51.44$ ,  $127.02 \pm 43.10$ ,  $180.50 \pm 81.24$  and  $258.73 \pm 114.46$ , respectively) and in the C group ( $67.54 \pm 47.18$ ,  $146.27 \pm 46.67$ ,  $97.63 \pm 68.65$  and  $309.74 \pm 311.58$ , respectively). The differences were significant ( $P < 0.05$ ).

The concentration levels of NE and IL-4 in group A were significantly higher than those in group C. The concentration levels of IFN- $\gamma$  in groups NA and A were  $307.81 \pm 73.52$  and  $285.88 \pm 91.23$ , respectively which were significantly lower than those of the group C ( $397.11 \pm 72.91$ ).



**Figure 2**

Comparison of the levels of cytokines in BALF. The concentration levels of ICOSL in the NA group were  $0.98 \pm 0.09$  and were significantly higher than those of the A group ( $0.33 \pm 0.09$ ) and the C group ( $0.43 \pm 0.21$ ,  $P < 0.05$ ). The mean concentration levels of NE, IL-17 and MMP9 in the NA group were  $37.70 \pm 6.93$ ,  $18.47 \pm 2.33$  and  $58.62 \pm 11.30$ , respectively, which were significantly higher than those of the A group ( $15.87 \pm 9.17$ ,  $9.59 \pm 3.52$  and  $40.16 \pm 18.19$ , respectively,  $P < 0.05$ ) and of the C group ( $27.33 \pm 6.01$ ,  $6.69 \pm 4.09$ ,  $27.66 \pm 15.74$ , respectively,  $P < 0.05$ ). The concentration levels of MMP9 in group A were

significantly higher than those in group C. The expression levels of IFN- $\gamma$  in group NA were  $489.66 \pm 88.08$ , which were lower than those in group A ( $706.37 \pm 106.33$ ) and those in group C ( $666.42 \pm 92.80$ ). The differences were significant ( $P < 0.05$ ). IL-17 levels in group NA were significantly higher than those in group C, whereas the concentration levels of IL-4 in groups NA and A were significantly higher than those in group C.