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ABSTRACT

Background: Neutrophilic asthma is characterized by the predominant infiltration of neutrophils in airway inflammation.

Objective: To explore the therapeutic potential of an antibody against the inducible T cell co-stimulator ligand (ICOSL) in a mouse model of neutrophilic asthma.

Methods: Female BALB/c mice were randomly assigned to different groups. They were then injected with ovalbumin (OVA)/lipopolysaccharides (LPS) to induce neutrophilic asthma. The mice were then treated with either anti-ICOSL (the I group), control IgG (the G group), or no treatment (the N group). Additionally, a control group of mice received vehicle PBS and was labeled as the C group (n=6 per group). One day after the last allergen exposure, cytokine levels were measured in plasma and bronchoalveolar lavage fluid (BALF) using ELISA. After analyzing and categorizing BALF cells, the lung tissues were examined histologically and immunohistochemically.

Results: Administering anti-ICOSL resulted in a significant decrease in the total number of inflammatory infiltrates and neutrophils found in BALF. Moreover, it led to a decrease in the levels of interleukin (IL)-6, IL-13, and IL-17 in both BALF and plasma. Additionally, there was an increase in IFN-γ levels in the BALF of asthmatic mice (p<0.05 for all). Treatment with anti-ICOSL also reduced lung interstitial inflammation, mucus secretion, and ICOSL expression in asthmatic mice.

Conclusion: The treatment of anti-ICOSL effectively improved lung interstitial inflammation and mucus secretion in mice with neutrophilic asthma by restoring the balance of Th1/Th2/Th17 responses. These findings indicate that blocking the ICOS/ICOSL signaling could be an effective way to manage neutrophilic asthma.

Keywords: Asthma; Inducible T Cell Co-Stimulator Ligand; Neutrophils; Pathology
INTRODUCTION

Bronchial asthma is a chronic inflammatory disorder of the airways involving various cellular components in children, exerting a significant burden on medical and health resources (1). During the pathogenic process of asthma, various types of inflammatory cells, such as eosinophils, macrophages, neutrophils, and lymphocytes, infiltrate into the airway interstitial tissues and these inflammatory infiltrates secrete cytokines and other inflammatory mediators, exacerbating the airway inflammation and remodeling (2). Earlier research has indicated that the imbalance of allergen-specific Th1 and Th2 responses may be crucial for the development of asthma as high levels of allergen-specific IgE and eosinophil proliferation and activation are observed in asthmatic patients and animals (3-5).

However, neutrophil infiltrates occur predominantly in the sputum, bronchoalveolar lavage fluid (BALF), and airway interstitial tissues of some asthmatic patients (6). Neutrophils are the shortest-lived terminally differentiated cells in the bloodstream and serve as the main inflammatory cells in the body. In *in vitro* experiments, numerous factors associated with inflammatory responses can influence the occurrence of neutrophil apoptosis. IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF) can impede the apoptosis of neutrophils (7). Neutrophils can also stimulate the production of IL-8, promoting their accumulation at the site of inflammation, further exacerbating airway inflammation, and leading to tissue damage. Emerging evidence from animal experiments and clinical trials indicates that IL-17 produced by Th17 cells can induce the secretion of CXCL1 and IL-8, promoting the recruitment of neutrophils to infiltrate the airways. This recruitment further leads to the expression of IL-6 and GM-CSF by neutrophils, resulting in an inflammatory response dominated by neutrophils (8, 9). Furthermore, this mechanism significantly contributes to the pathogenesis and exacerbation of asthma, particularly in cases of severe asthma and steroid-refractory asthma. Unfortunately, there are currently no viable treatment options for addressing neutrophilic asthma in clinical practice. Hence, the discovery of a new therapeutic strategy for neutrophilic asthma will be of significance in the management of asthmatic children.

Co-stimulatory signals are crucial for the T cell activation (10). Inducible T cell co-stimulator ligand (ICOSL) is a member of the CD28/B7 superfamily (11), and ICOSL can engage the ICOS receptor on immunocytes to activate the ICOS/ICOSL signaling. ICOS/ICOSL signaling is vital for sustaining T lymphocyte activation and function by promoting antigen-driven T cell proliferation, cytokine secretion, immunoglobulin class switching, and modulating the functional differentiation of Th1, Th2, and Th17 cells (12-15). Activation of the ICOSL/ICOS signaling can also facilitate the generation of cytokines, including IL-4, IL-6, and IL-17 (16), and enhance inflammation. Cytokines secreted by T cells, such as IL-6, IL-10, and IL-23, play a critical role in the mobilization, recruitment, and activation of neutrophils (17). However, whether the blockage of the ICOSL/ICOS signaling can modulate neutrophilic asthma has not been clarified. Currently, neutrophilic asthma is highly anticipated because of its severity, difficulty to be treated, and hormone insensitivity (18). Neutrophilic asthma has become a difficult problem in the treatment of asthma, and it is an urgent problem to be solved. In this research, we utilized a mouse model of ovalbumin (OVA)/lipopolysaccharides (LPS)-induced neutrophilic asthma to test the therapeutic potential of anti-ICOSL antibody during the induction period on neutrophilic asthma and airway inflammation. This study aimed to explore the potential of blocking the ICOSL/ICOS signaling for the management of refractory and severe neutrophilic asthma in the clinic.
MATERIAL AND METHODS

A Mouse Model of Neutrophilic Asthma

A total of twenty-four female BALB/c mice (6-8 weeks old, weighing 20±2g) were procured from the Animal Laboratory Center of Suzhou University. These mice were housed in a specific pathogen-free facility maintained at a constant temperature of 22±2℃, with a humidity level of 50~60%, and a 12-hour light-dark cycle. The mice were provided ad libitum access to mouse chow and water. Subsequently, the mice were randomly assigned to four groups, with six mice per group. The mice were injected intraperitoneally with vehicle PBS (0.2 ml) or OVA+LPS suspension (50 µg OVA and 10 µg LPS) on days 0, 7, and 14, respectively. Some of the OVA+LPS sensitized mice were injected into the peritoneal cavity with vehicle PBS as the N group, 50 mg/Kg anti-ICOSL monoclonal antibody (HK5.3, eBioscience, San Diego, CA, USA) or the isotype control IgG (Biolegend, San Diego, CA, USA) on days 0, 3, 7, 10, and 14 as the I or the G group, respectively. Beginning on day 22 post sensitization, the PBS-injected mice were continually challenged daily for seven consecutive days with 0.2 ml of PBS that had been ultrasonic-atomized for 30 min as the C group. The OVA-LPS-sensitized mice were challenged with 1% OVA that had been ultrasound-atomized for 30 min for seven consecutive days (Fig. 1). The experimental protocol was approved by the Ethics Committee of the Children’s Hospital of Soochow University (approval number: 2019DW001). Furthermore, the study was conducted following the National or International Guidelines for the care and use of animals.

Collection and Treatment of Specimens

After general anesthesia, the blood samples were collected from individual mice using a standard mouse orbital sinus collection method (19). The blood samples in the EDTA-contained tubes were centrifuged to prepare plasma samples. The left lung of each mouse underwent lavage with 0.5 ml of PBS, repeating the process three times. The collected BALF samples (about 1.5 mL/mouse) were centrifuged and the pelleted cells were suspended in 100 µl of PBS for cell analysis. The cell samples were mounted on slides, dried, and stained with a mixture of methylene blue and Eosin Y. The total number of cells and their classification were microscopically examined (Olympus, Tokyo, Japan). The plasma and BALF samples were subsequently

Fig. 1. The protocol for inducing a mouse model of neutrophilic asthma. OVA: ovalbumin; PBS: phosphate buffered saline; LPS: lipopolysaccharides; ICOSL: inducible T cell co-stimulator ligand
stored at -80℃ until further analysis. Their lung tissues were collected for histology and immunohistochemistry analyses.

**Enzyme-linked Immunosorbent Assay (ELISA)**

The concentrations of IL-6, IFN-γ, IL-13, and IL-17 in the plasma and BALF samples were determined using specific ELISA kits (R&D Systems, Minnesota, USA) following the manufacturer’s instructions.

**Pathological Changes in Lung Tissue**

The lung tissue samples underwent overnight fixation in 10% formalin, followed by subsequent embedding in paraffin. The lung tissue sections (4 µm) were dewaxed, rehydrated, and routine-stained with hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS), respectively. The tissue section images were obtained using a microscope (Olympus). The pathological changes and inflammatory infiltrates were then evaluated.

**Immunohistochemistry of Lung Tissue**

The 4 µm lung tissue sections were dewaxed, rehydrated, and then treated with 3% methanol to inactivate endogenous peroxidase. The sections were incubated...

**Fig. 2.** The total count and classification of cells in BALF were analyzed in each group. The N group exhibited the highest total cell count in the BALF of mice among the different groups, followed by group I and group G, and the lowest cell count was observed in group C (p<0.05). The group with the lowest share of neutrophils in BALF was group C, the highest was in group N and group G (p<0.05). BALF: bronchoalveolar lavage fluid.
overnight at 4°C with anti-ICOSL (599841, eBioscience), and after washing, the bound antibodies were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies (Shanghai Dingji Biotechnology, Shanghai, China). The visualization was achieved via DAB (3,3’-Diaminobenzidine). The tissue section images were obtained using a microscope (Olympus) and the levels of anti-ICOSL staining were analyzed with Image-Pro Plus 6.0 software in 10 fields selected randomly from each section (magnification ×400) using the unstained area as the control.

Statistical Analysis

The data were represented as the mean±standard deviation (SD). Shapiro-Wilk tests were utilized to check the normality of the data. The difference among the groups was analyzed by Analysis of Variance (ANOVA) where applicable using SPSS18.0 software. A p-value of <0.05 was considered statistically significant.

RESULTS

Analysis of Inflammatory Infiltrates in BALF of Mice

All mice demonstrated a BALF recovery rate exceeding 80%, with an average recovery rate of (87.40±3.37%). Regarding the N group, the total cell count in the BALF of mice was higher than those in other groups of mice (p<0.05 for all) and the total number of cells in the I and G groups was also significantly higher than those in the C group (p<0.05 for all, Fig. 2). The neutrophil percentages in the N group BALF were comparable with those in the G group and significantly higher than those in the I and C groups. (p<0.05 for all, Fig. 2). Furthermore, the percentages of neutrophils in BLAF from the I group of mice remained markedly elevated than those in the C group (p<0.05). In contrast, the percentages of eosinophils, lymphocytes, and neutrophils in BALF from the N, I, and G groups were markedly elevated compared with the C group. Hence, treatment with anti-ICOSL mitigated the neutrophil infiltration in the lungs of mice.

The Levels of Cytokines in Plasma and BALF

Compared with the concentrations of plasma IFN-γ in the C group, the concentrations of plasma IFN-γ significantly decreased in asthmatic mice regardless of anti-ICOSL treatment (p<0.05 for all) and there were no statistically significant differences in the plasma levels of IFN-γ among the N, I, and G groups of mice (Fig. 3A). In contrast, the plasma concentrations of IL-6, IL-13, and IL-17 measured in the N group showed a significant increase and were noticeably higher compared with those in the I and G groups, while there was no difference in plasma levels of IL-13 between the N and G groups (p<0.05 for all). Moreover, the levels of plasma IL-6 in the G group were significantly higher than that in the I group (p<0.05, Fig. 3A).

Similarly, compared with the levels of BALF in the C group, the levels of IFN-γ in the BALF of the N group significantly decreased and were comparable with that in the G group, which showed a noteworthy reduction in comparison with the I group (p<0.05 for all, Fig. 3B). Conversely, the N group exhibited considerably higher BALF concentrations of IL-6, IL-13, and IL-17, with IL-6 and IL-17 levels resembling those in the G group, but noticeably more elevated than in the I group (p<0.05 for all). Collectively, the data indicated that anti-ICOSL treatment modulated systemic and local inflammatory responses by enhancing Th1 responses but reducing Th17 responses in asthmatic mice (Fig. 3B).

Histopathological Analysis

To understand the effect of anti-ICOSL, the lung tissues from the various cohorts of mice underwent H&E staining. As shown in Fig. 4, there was no obvious abnormality or inflammatory cell in the lung tissues of the C group of mice (Figs. 4a and b).
Fig. 3. The cytokine levels in both plasma and BALF. Group C exhibited a significantly higher concentration of IFN-γ in plasma compared with group N, group I, and group G ($p<0.05$). Among the different groups, the lowest concentration of IL-6 in plasma was observed in group I, while the highest concentration was found in the N group ($p<0.05$). Group C exhibited a notably lower cell count concentration of IL-13 compared with the N group, I group, and G group ($p<0.05$). The N group displayed a significantly higher concentration of IL-17 compared with group C, group I, and group G ($p<0.05$, Fig. 3A). In terms of the concentration of IFN-γ in BALF, group C exhibited the highest levels, significantly higher than those observed in group N and group G ($p<0.05$). The IL-6 level was the lowest in group C ($p<0.05$). Group C had the lowest IL-13 level, whereas group N exhibited the highest level. The IL-13 levels in groups I and G were lower compared with those in group N ($p<0.05$). Group N had the highest IL-17 levels, while group C, group I, and group G all had lower IL-17 levels compared with group N ($p<0.05$, Fig. 3B). BALF: bronchoalveolar lavage fluid, IL: interleukin
In contrast, in the N (Figs. 4c and d) and G (Figs. 4g and h) groups, numerous inflammatory infiltrates, likely composed of neutrophils, lymphocytes, and plasma cells, were observed in the lung. Additionally, tracheal stenosis, edema in the bronchial epithelium, shedding and degeneration, as well as inflammation in the peritracheal lung tissues and alveolar septum were observed in the N (Figs. 4c and d) and G (Figs. 4g and h) groups of mice. Such lung pathological changes were ameliorated in the I group of mice (Figs. 4e and f). PAS staining suggested that there was much mucus in the alveolar space of the N (Figs. 5c and d) and G groups (Figs. 5g and h) of mice, but not in the C group (Figs. 5a and b). The I group of mice exhibited a noticeable reduction in PAS-stained mucus (Figs. 5e and f) in contrast to the N and G groups. Immunohistochemistry analysis revealed that while there was no obvious anti-ICOSL staining in lung tissue sections from the C group of mice (Figs. 6a and b) strong anti-ICOSL staining signals
were detected in lung tissue sections from the N (Figs. 6c and d) and G (Figs. 6g and h) groups. However, the anti-ICOSL staining signals reduced in lung tissue sections from the I group (Figs. 6e and f) of mice, compared with that in the N and G groups. Quantitative analysis unveiled that the anti-ICOSL signals in asthmatic mice were significantly higher than that in the C group \((p<0.05\) for all, Table 1). The N and G groups of mice exhibited significantly higher levels of anti-ICOSL signals compared with the I group, while the levels between the N and G groups were similar \((p<0.05\) for both). Together, the results indicated that anti-ICOSL treatment significantly mitigated inflammation and mucus secretion in the lungs of asthmatic mice.

**DISCUSSION**

New findings suggest that in some patients with severe asthma and acute exacerbations,
**ICOSL, a novel target for neutrophilic asthma?**

*Fig. 6.* Evaluation of ICOSL expression in lung tissues from various groups of mice using immunohistochemistry analysis. Immunohistochemistry using anti-ICOSL antibody was performed to determine the levels of ICOSL expression in lung tissues from the different groups of mice. Representative images (magnification ×400) were obtained from the different groups (n=6 per group) of mice from two separate experiments to reduce repetition. a and b: The C group; c and d: The N group; e and f: The I group; g and h: The G group. Bar=100 µm. Brownish yellow indicates positive anti-ICOSL staining. ICOSL: inducible T cell co-stimulator ligand.

**Table 1. ICOSL expression in lung tissue (mean gray value, IOD)**

<table>
<thead>
<tr>
<th>Group</th>
<th>ICOSL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C group</td>
<td>6.77±2.75</td>
</tr>
<tr>
<td>N group</td>
<td>75.62±10.52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>I group</td>
<td>48.71±13.32&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>G group</td>
<td>70.31±13.65&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>P</em></td>
<td>0.002</td>
</tr>
</tbody>
</table>

<sup>a</sup>Compared with group C, *p*<0.05; <sup>b</sup>Compared with group N, *p*<0.05; <sup>c</sup>Compared with group I, *p*<0.05

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neutrophil infiltration can occur in both the lung interstitial tissues and induced sputum samples, even in cases where typical eosinophil infiltration is absent (20-22). Neutrophils are terminally differentiated cells with a short half-life due to their spontaneous apoptotic process and function to be important defenders against bacterial infection. During the process of asthma, inflammation-induced damage in the respiratory tract can enhance tissue repairing and remodeling. Activated neutrophils can secrete many inflammatory mediators to promote inflammation, leading to airway epithelial tissue damage. During the acute asthmatic attack, significant elevation of IL-6, IL-8, and IL-17 levels was observed in both peripheral blood and BALF. These cytokines play a role in inhibiting neutrophil apoptosis and facilitating their accumulation within the inflamed airways. (23-25). Due to its key involvement in T cell activation, Th1/Th2 response polarization, and immunoglobulin isotype switching, the ICOS/ICOSL signaling pathway represents a promising therapeutic target for modulating inflammatory responses. ICOSL deficiency can reduce serum IgE levels and limit airway inflammatory infiltration in rodents following asthmatic induction. ICOS defect affects germinal center formation, antibody classification switching, and IL-4, IL-13 production in mice (26). Treg amplification can inhibit the production of ILC2 cells through the ICOS/ICOSL pathway, alleviating allergic lung inflammation (27). These findings suggest that ICOSL may play a crucial role in the pathogenesis of asthma.

In this study, we established neutrophilic asthma in mice by continually sensitizing them to OVA+LPS and subsequent OVA challenges. There were predominant neutrophil infiltrates in lung tissues, BALF, and around the trachea, accompanied by the presence of massive mucus in the trachea, tracheal stenosis, and higher levels of pro-inflammatory cytokines of IL-6, IL-13, and IL-17 in both plasma and BALF. These data suggest that this neutrophilic asthmatic model may be valuable for drug screening and pathological research.

In this study, we found that treatment with anti-ICOSL during the sensitizing period significantly delayed and ameliorated the allergen-stimulated asthmatic behaviors in mice. These data indicated that the blockage of the ICOSL/ICOS signaling alleviated the allergy-stimulated clinical symptoms in neutrophilic asthmatic mice and also suggested that this signaling participated in the initiation, progression, and acute exacerbation of neutrophilic asthma.

A previous study has shown that the ICOSL/ICOS signaling contributes to the development and progression of inflammation, tumors, and autoimmune diseases (28). In areas of inflammation, ICOSL is expressed at high levels and its upregulation strongly correlates with the extent or intensity of the inflammation. The function of ICOSL in the pathogenesis of asthma has not been clarified. A study has shown that transferring OVA-reactive T cells expressing ICOS into BALB/c mice sensitized to OVA results in increased infiltration of lymphocytes, macrophages, neutrophils, and eosinophils in their BALF (29), while another study points out that the activation of the ICOSL/ICOS signaling enhances Treg responses in asthmatic mice (30). Elevated expression of ICOSL by pulmonary dendritic cells is critical for the expansion of Tregs (31). A commonly recognized fact is that Tregs can prevent the activity of antigen-specific T cells and airway hyperresponsiveness. (32). In this study, we observed that anti-ICOSL treatment significantly attenuated the expression of IL-6, IL-13, and IL-17, but increased IFN-γ in both plasma and BALF of mice, compared with the untreated asthmatic mice. These data suggest that blockage of the ICOSL/ICOS signaling may enhance Th1 responses, but attenuate Th2 and Th17 responses, ameliorating their imbalance to mitigate the pathological and immune inflammation cascade observed in neutrophilic asthma. It is possible that blockage of the ICOSL/ICOS signaling may
attenuate Treg responses to enhance Th1 responses during the process of neutrophilic asthma. Alternatively, the decreased Th2 and Th17 responses suggest that their activation and functions may be more dependent on the ICOSL/ICOS signaling in neutrophilic asthmatic mice. Our interest lies in examining the regulatory function of anti-ICOSL in Treg responses and assessing its potential for ameliorating neutrophilic asthma.

CONCLUSION

In summary, the results indicated that treatment with anti-ICOSL before allergen challenges significantly mitigated inflammatory cell infiltration and mucus secretion in the lungs of neutrophilic asthmatic mice. Mechanistically, anti-ICOSL treatment significantly diminished the concentrations of IL-6, IL-13, and IL-17 in plasma and BALF, but increased IFN-γ in BALF of neutrophilic asthmatic mice to modulate the imbalance of Th1, Th2, and Th17 responses. These findings imply that inhibiting the ICOS/ICOSL signaling may be valuable for suppressing neutrophilic asthma and the ICOS/ICOSL signaling may be a new target for the treatment of asthma.

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AUTHORS’ CONTRIBUTION

CH and ZC designed the study. HD and YR performed all the experiments and wrote the manuscript. YS and WJ aggregated the data. YY, CZ, LH, MW, WG, XZ, and HS analyzed the data. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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