

Up-regulation of CD44 expression by interleukin-13 in a murine model of asthma

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Abstract. Allergic asthma is a chronic inflammatory disease characterized by the infiltration of inflammatory cells, the elevated production of cytokines and enhanced airway hyper-reactivity (AHR). CD44 is a widely expressed cell adhesion molecule that is involved in lymphocyte adhesion to inflamed endothelium. Previous studies showed that pulmonary eosinophils and CD4⁺ T cells express high levels of CD44 in bronchoalveolar lavage fluid following antigen administration. The aim of this study was to investigate whether interleukin (IL)-13 is capable of modulating CD44 expression in lung tissue and lymphocytes, further promoting inflammatory cell recruitment into the lungs and exacerbating asthmatic responses. Six- to eight-week-old male Balb/c mice were used. Quantitative PCR and immunohistochemistry were employed to determine the effect on CD44 expression. Our findings showed that IL-13 may play a significant role during the challenge phase in that CD44 expression was up-regulated in lung tissue and lymphocytes following IL-13 treatment, resulting in inflammatory cells infiltrating into lungs and enhancing AHR.

Introduction

Asthma is a chronic disease affecting an estimated 300 million people worldwide (1). It is a chronic inflammatory disease characterized by airway inflammation, including infiltration of inflammatory cells, elevated production of cytokines and enhanced airway hyperreactivity (AHR) (2).

Eosinophil and T-lymphocyte (CD4⁺) infiltration and the increased secretion of inflammatory mediators during asthma adversely affect the large and small airways (3). Currently, novel

therapeutics to treat chronic pulmonary inflammatory diseases, such as asthma, are under investigation. Pro-inflammatory cells (e.g., mast cells, eosinophils and neutrophils), cytokines and other mediators found in the airways are key targets for drug development.

Lymphocytes are predominant among inflammatory cells infiltrating the asthmatic airways, and cell-mediated immunity may play a significant role in the pathogenesis of asthma. Since peripheral blood is the reservoir of lymphocytes that are recruited into the bronchial tree, it is of interest to investigate activated T cells and cytokines in the peripheral blood of asthmatic patients. Increased numbers of activated T cells are found in the peripheral blood during acute episodes of asthma (4).

Other investigators previously reported that CD4⁺ T cells expressing high levels of CD44 accumulate in the lungs following antigen administration in a murine model of asthma (5). CD44 is a widely expressed cell adhesion molecule that is involved in lymphocyte adhesion to inflamed endothelium and tumor metastasis, and serves as a surface binding site for a number of cytokines and signaling cells, such as eosinophils, lymphocytes and macrophages infiltration (6).

Studies of mouse allergic airway disease models also show that interleukin (IL)-13, a cytokine produced by Th2 cells and other cells recruited to the lungs during allergic responses, is required for AHR, mucus secretion and allergen-induced airway inflammation (7,8). IL-4 and IL-13 have been found to induce the up-regulation of CD44 expression in various cell lines (9-11). We examined cytokine IL-13 and the cell adhesion molecule CD44 due to their putative relationship to the pathobiology of asthma.

The aim of the present study was to investigate the effect of IL-13 on CD44 expression in asthma and to determine whether IL-13 contributes to the infiltration of inflammatory cells through alteration of CD44 expression.

Materials and methods

Animals. Six- to eight-week-old specific pathogen-free male Balb/c mice (weight 20-22 g) were purchased from the Experimental Animal Center in the Second Affiliated Hospital of Harbin Medical University (Harbin, China). The mice were kept in conventional pathogen-free housing and were maintained on OVA-free diets. Experiments were performed according to protocols approved by the Animal Studies Committee of China.

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Table I. Sequences of specific primers.

Gene	Primer sequence	Length (bp)
CD44		
Forward	5'-CACCATCGAGAAGAGCACCCCAGAA-3'	393
Reverse	5'-TCTGTTTCATCTTCATTTTCCTC-3'	
GAPDH		
Forward	5'-AAGGTCGGTGTGAACGGATTGG-3'	239
Reverse	5'-TTAGTGGGGTCTCGCTCCTGGAA-3'	

Mouse model of asthma. The protocol for inducing experimental asthma was previously described (12,13). Treated mice exhibit features reminiscent of human asthma, including eosinophilic airway inflammation and AHR. In brief, groups of mice (n=6) were sensitized by intraperitoneal (i.p.) injection of 100 μ g OVA (Sigma Chemical Co., St. Louis, MO, USA) absorbed in 2 mg alum on alternate days from Days 1 to 14. The mice were then challenged via the airways with OVA (3 mg/ml) in phosphate-buffered saline using an ultrasonic nebulizer for 30 min daily from Days 15 to 29 (OVA-treated group). Control mice (Control) were injected with 0.5 ml of sterile saline and then exposed to aerosolized sterile saline using similar equipment and schedules.

Mouse recombinant IL-13 (R&D Systems, Minneapolis, MN, USA) (100 ng in 0.25 ml of saline) was intravenously administered 1 h after the last OVA/saline challenge (OVA + IL-13 and saline + IL-13-treated groups). Analyses were performed 24 h after the final airway challenge.

Airway responsiveness measurements. Airway reactivity was measured *in vivo* 24 h after the last aerosol exposure. Mice were anesthetized with an i.p. injection of urethane (1,300 mg/kg) and were placed in a whole-body plethysmography chamber. Lung resistance (R_L) was measured at baseline. Methacholine concentrations of 6.25, 12.5, 25 and 50 mg/ml were then administered intravenously at 4-min intervals, and dose-response curves were obtained.

Bronchoalveolar lavage. Following the AHR measurements, the right lung was lavaged six times with 1 ml D-Hang's solution. Total bronchoalveolar lavage fluid (BALF) was centrifuged (1,000 g for 10 min at 4°C). The cell pellet was resuspended in D-Hang's solution. Cytospin preparations were stained with Wright-Giemsa. Differential cell counts were performed using morphological criteria. After BAL sampling, left lung tissue was removed, fixed with 10% formalin, and paraffin-embedded sections were generated. Approximately 200 mg of lung tissue was dissected, snap-frozen and stored in liquid nitrogen. The supernatant was collected for cytokine analysis and the cell pellet.

Lymphocyte isolation. Mouse blood samples were collected by cardiac puncture, and mononuclear cells were isolated by gradient centrifugation on Histopaque-1083 (Sigma Chemical) according to the manufacturer's instructions. The mononuclear cell layer was carefully collected and the cells were stimulated with 10 mg/ml Con A (Sigma Chemical) in

10% FBS for 3 h. The unadhered lymphocytes were harvested and stained with fluorescent antibodies against CD3 and CD19 (BD Pharmingen, San Diego, CA, USA), followed by examination under a fluorescent microscope. The isolated lymphocytes used in our experiments had a purity of >90%.

Quantitative real-time PCR on lung tissue and lymphocytes. Total RNA was extracted from 100 mg of lung tissue and 5×10^5 lymphocytes/ml using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), and then RNA was reverse transcribed into cDNA using Strata Script™ reverse transcriptase (Stratagene, La Jolla, CA, USA). GAPDH was used as a control gene. The levels of CD44 and GAPDH mRNA transcripts were characterized by quantitative RT-PCR using the SYBR-Green PCR Core reagents kit (Applied Biosystems, USA) and specific primers on a DA7600 PCR amplifier (DAAN, China). The primers were designed according to the reported gene sequences of mouse CD44 (GenBank accession no. AJ251594) and GAPDH (NM 008084). The sequences of specific primers are shown in Table I. The PCR reactions (50 μ l/tube, in duplicate) were denatured at 94°C for 2 min and subjected to 30 cycles of 94°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec. The relative levels of mRNA transcripts were analyzed by normalizing the values of individual samples to GAPDH.

Immunohistochemical staining for CD44. The protocol for immunostaining was used in the same manner as a previously published method (6,14). Either rabbit anti-mouse CD44 or rabbit anti-mouse β -actin antibody (Wuhan Boster Bio-engineering Co., Ltd., China; 1:200 dilution) was used. Sections were reacted with ABC reagent and diaminobenzidine for visualization. As a negative control, sections were stained without the presence of primary or secondary antibodies. Sections were examined in a blind manner under a light microscope. Expression of CD44 was considered positive when a brown membrane staining pattern was observed. CD44 expression was graded on infiltrated inflammatory cells and airway smooth muscle and alveolar epithelium from asthmatic mice semiquantitatively on the following scale: +++, strong; ++, moderate; +, mild; \pm , weak; -, undetectable.

Data analysis. Data were analyzed using the SPSS statistical program. Values were expressed as the means \pm SD. Statistical comparisons were conducted by t-test and ANOVA. Data indicate statistical significance at $p < 0.05$ or $p < 0.001$.

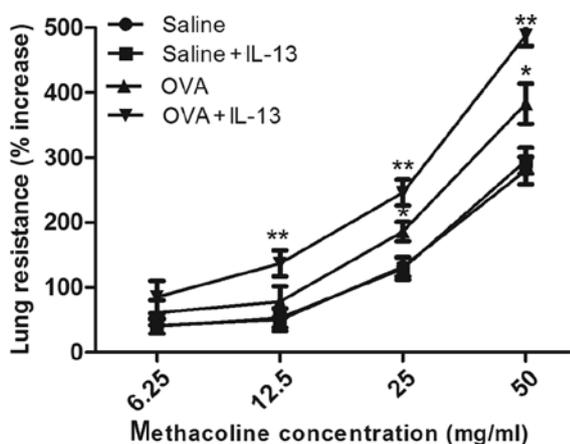


Figure 1. Administration of recombinant IL-13 increases OVA-induced AHR. Saline/OVA-challenged mice (n=6 per group) were injected with recombinant IL-13 at 1 h after the final challenge. Data are the means \pm SD. * p <0.05, OVA-treated vs. saline-treated groups; ** p <0.05, OVA-treated group vs. OVA + IL-13-treated group.

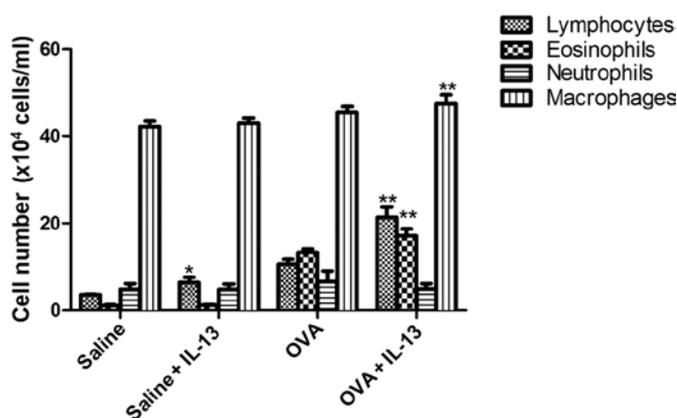


Figure 2. Increased inflammatory response in mice treated with recombinant IL-13 on the number of inflammatory cells in BALF. Saline/OVA-challenged mice (n=6 per group) were treated with recombinant IL-13 at 1 h after a final challenge. The degree of inflammatory cell infiltration in the lungs was detected. Data are the means \pm SD. * p <0.05; saline-treated vs. saline + IL-13-treated groups; ** p <0.05 or p <0.001. OVA-treated vs. OVA + IL-13-treated groups.

Results

Administration of recombinant IL-13 increases OVA-induced AHR. To test the ability of IL-13 to increase AHR in the asthmatic model, R_L responding to methacholine was employed. The OVA-challenged mice developed significant AHR compared to the control mice, as evidenced by an increase in R_L (Fig. 1). Administration of IL-13 further increased R_L in the OVA-challenged mice, suggesting the increase of AHR (p <0.05). However, the saline + IL-13-treated mice did not find a measurable effect on AHR comparison to control mice. These data suggested that administration of IL-13 further promotes OVA-induced AHR.

Effect of recombinant IL-13 on the number of inflammatory cells in BALF. Previous studies showed that following the sensitization and challenge with OVA, the number of total inflammatory cells was increased significantly in BALF (13).

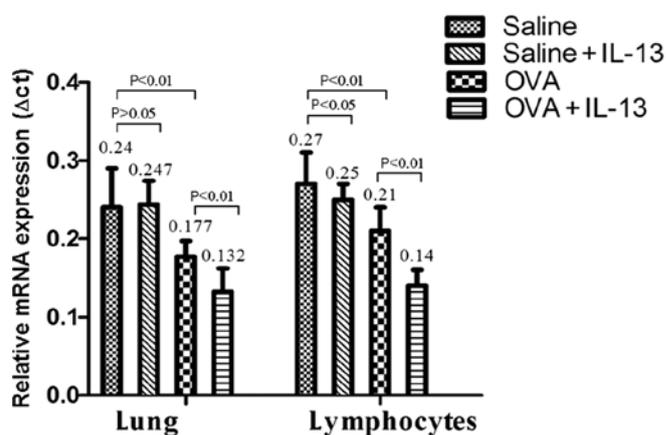


Figure 3. Effect of recombinant IL-13 on CD44 expression in mouse lung tissue and lymphocytes by quantitative RT-PCR. Mouse recombinant IL-13 (100 ng) was administered at 1 h after the final challenge. The relative levels of CD44 mRNA transcripts were determined by quantitative RT-PCR.

Table II. CD44 expression in the lung tissue of mice in different groups.

Groups	No.	Expression of CD44 (means \pm SD)	F	p-value
Saline	6	1.06 \pm 0.20		
Saline + IL-13	6	1.50 \pm 0.23	1.86	>0.05
OVA	6	2.86 \pm 0.20	36.25	<0.01 ^a
OVA + IL-13	6	3.53 \pm 0.40	11.35	<0.01 ^b

Response level +++, 4; ++, 3; +, 2; \pm , 1; -, 0; * p <0.01, OVA-treated vs. saline-treated groups; ^b p <0.01, OVA + IL-13-treated vs. OVA-treated groups.

In this study, we further investigated the number of inflammatory cells following the administration of mouse recombinant IL-13 during the OVA challenge. The results showed that the number of lymphocytes, eosinophils and macrophages was increased significantly compared to the OVA-treated mice. However, in the saline + IL-13-treated mice, the number of inflammatory cells was increased only in lymphocytes (p <0.05) compared to that observed in the control mice (Fig. 2). These results showed that IL-13 promotes inflammatory cell (especially in lymphocyte) infiltration into the lungs during the challenge phase.

IL-13 up-regulates CD44 expression in the lung tissue and mouse lymphocytes. To further examine the increase of CD44 expression following the administration of IL-13, we investigated the mRNA expression of CD44 in the lung tissue and peripheral blood lymphocytes by quantitative RT-PCR. We found that CD44 expression in the lung tissue and lymphocytes was increased in the OVA-challenged mice compared to the control mice (p <0.01). In the saline + IL-13-treated group, CD44 expression was increased in peripheral blood lymphocytes, but not in the lung tissue. However, in the OVA + IL-13-treated group, CD44 expression was significantly increased in

the lung tissue and peripheral blood lymphocytes compared to the OVA-treated mice (Fig. 3).

Since it has been reported that pulmonary eosinophils and CD4⁺ T cells expressed higher levels of CD44 in BALF (15), we examined CD44 protein expression in lung by immunohistochemistry. Immunohistochemistry for CD44 revealed that only a few CD44-staining cells were present in the lungs of the control mice. In the saline + IL-13-treated group, CD44 protein expression was increased mildly in the lung tissue ($p > 0.05$). In the OVA-challenged mice, CD44 immunoreactivity was observed in certain mononuclear cells infiltrating the airways and airway smooth muscle, and in the alveolar epithelium of asthmatic mice. Notably, after treatment with IL-13, the quantity of CD44-expressing in lung tissues was significantly increased (Table II).

Discussion

The pathophysiology of asthma involves complex interactions between multiple inflammatory mediators and the influx of numerous inflammatory cell types. Furthermore, studies on eosinophils have clearly demonstrated that cytokine-mediated activation resulted in *de novo* induction and/or up-regulation of the surface expression of several molecules (16).

Studies have found that IL-4 and IL-13 induce the up-regulation of CD44 expression in cell lines derived from the colon and human cervical adenocarcinoma (9-11). However, whether the increased CD44 expression in lung tissue and lymphocytes is induced by IL-13 in asthma remains to be clarified. The objective of this study was to determine the effect of IL-13, a Th2 cytokine, on CD44 expression during a murine model of asthma. Strategies to directly block CD44 function may result in the decrease of asthma progression.

In the present study, we investigated for the first time whether OVA sensitization and challenge up-regulate the expression of CD44 in lung tissue and leukocytes, and whether the delivery of IL-13 is capable of significantly increasing this change. Our data also demonstrate that the inflammatory effects of IL-13 during the challenge phase are associated with increases in CD44 expression, AHR and inflammatory cell infiltration in the lungs, which can lead to other pro-inflammatory effects.

In allergic asthma, CD4⁺ T lymphocytes are a fundamental component of local chronic inflammation (17). The circulating cytokine profile of these cells is oriented towards a Th2 phenotype, characterized by the production of IL-4, IL-5 and IL-13. The findings of Katoh *et al* (15) support an essential role for CD44 in allergen-induced Th2 cell and eosinophil recruitment to the lungs. Li *et al* also reported that the percentage of CD44-positive leukocytes showed a significant correlation with several important indices of asthma, including the number of lung inflammatory cells, levels of IL-4 and IL-5 in BALF, and hyperplasia of airway goblet cells (14). The presence of adhesion molecules on airway epithelial cells may be important in recruiting leukocytes to the epithelium. A previous study investigated the effects of IL-4, IL-13 and interferon- γ on intracellular adhesion molecule-1 and zonula occludens protein-1 expression on cultured human airway epithelial cells (18).

We have shown that a single intravenous administration of IL-13 in this challenge model further promotes airway inflammation as well as the up-regulation of CD44 expression in the

lungs and lymphocytes (Figs. 2 and 3). Moreover, the administration of anti-CD44 mAb in the asthma model prevented the accumulation of leukocytes in the lungs and subsequently blocked AHR (15), suggesting that CD44 contributes to allergic airway inflammation in the effector phase of asthma.

However, our results contrast with the findings reported by Teder *et al* (19). In that study, CD44^{-/-} mice developed exaggerated lung inflammatory responses in a bleomycin-induced model of lung injury. Instead, our study agrees with the previously mentioned findings reported, which demonstrate an anti-CD44 antibody reduction of allergen-induced AHR and airway inflammation in sensitized mice and an associated reduction in allergen-induced IL-13 (20). These studies showed the correlation of IL-13 level and CD44 expression in allergic asthma.

In this study, we found that OVA-challenged mice induced an increase of CD44 expression in lung tissue and lymphocytes compared to the control group. Moreover, in the OVA + IL-13-treated mice, CD44 expression was increased significantly in the lung tissue and lymphocytes compared to the control or the OVA-challenged mice as measured by quantitative real-time PCR and immunohistochemistry (Fig. 3 and Table II). However, no difference was found in the CD44 expression in the lung tissue of the saline + IL-13-treated mice compared to the control mice. These studies demonstrate that OVA sensitization and challenge up-regulates the expression of CD44 in lung tissue and lymphocytes, and that the delivery of IL-13 significantly increases this change. This change of CD44 expression on peripheral blood lymphocytes may be attributable to the circulating IL-13 levels, since high levels of CD44 expression on lymphocytes may be induced by IL-13.

In conclusion, our study suggests that IL-13 treatment increased asthmatic responses and was associated with the up-regulation of CD44 expression in lung tissue and lymphocytes. Treatment with IL-13 may up-regulate CD44 expression and therefore increase the infiltration of leukocytes in the lungs, thereby increasing the asthmatic response.

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