

Brief report

Overexpression of *mc1ca3* in airway epithelium of asthmatic murine models with airway inflammation

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Keywords: *asthma; airway epithelial cell; mc1ca3; inflammation*

Asthma is a worldwide prevalent disease that is a considerable health burden in many countries.¹ In recent years, the airway epithelium is increasingly recognized as a central contributor to the pathogenesis of asthma.² One of the most highly induced genes in epithelial cells in experimental allergic airway disease is the third murine calcium-activated chloride channel homologue (*mc1ca3*, alias *gob-5*). Its human homology protein is *hCLCA1*,^{3,4} which has been identified as clinically relevant molecules in diseases with secretory dysfunctions including asthma and cystic fibrosis. In initial studies, *mc1ca3* was thought to be a member of calcium-activated chloride channel (CaCCs) family, whereas some new interesting reports suggest that the two *mc1ca3* cleavage products cannot form an anion channel on their own but may instead act as extracellular signaling molecules with as yet unknown functions and interacting partners.⁵

Studies on the effect of *mc1ca3* in asthmatic mice showed conflicting results, and the controversy has been centered on goblet cell hyperplasia and mucus production associated with *mc1ca3*.^{6,7} By contrast, few studies about its effects on immune or inflammatory response can be found. A new finding was that *mc1ca3* was involved in regulating the tissue inflammation in innate immune response.⁸ As we know, allergen exposure stimulates eosinophilic inflammation of the airways associated with infiltration of T cells in asthma.⁹ Therefore, we presume *mc1ca3* is involved in regulating the tissue inflammation by affecting the recruitment of eosinophils and T cells in lung. However, there is no more evidence to clarify the effects and possible mechanisms of *mc1ca3* on lung inflammatory response in asthma.

From previous reports, it is not known whether *mc1ca3* production is an effect caused by the respiratory allergic inflammation or whether the levels of *mc1ca3* precede the development of allergic inflammation. Therefore, our current study was designed to explore the relative of *mc1ca3* and inflammatory response in asthma model and to find some new clues for our further study.

METHODS

Experimental animals

Sixteen six to eight-week-old specified-pathogens free

BALB/c mice (female) were purchased from the Center of Experimental Animals, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). These mice were randomly divided into two groups: control group (saline-sensitized on day 0 and day 14, saline-challenged from day 21 to day 23, *n*=8); Ovalbumin (OVA) group (OVA-sensitized on day 0 and day 14, OVA-challenged from day 21 to day 23, *n*=8). The asthmatic murine models were established as previously described.¹⁰ Twenty-four hours after the last challenge, the mice were sacrificed and the bronchoalveolar lavage (BAL)-fluid (BALF) and lung specimens were collected.

Bronchoalveolar lavage

Whole lung lavage was performed for each mouse, and 0.7 ml of room temperature sterile saline was infused into the lungs to total lung capacity twice, and then collected. The cells were then isolated from the whole lung lavage fluid by centrifugation at 1500 r/min for 10 minutes. The supernatant was stored at -80°C for later assessment of cytokine levels by enzyme-linked-immunosorbent assay (ELISA).

Differential cell counts of BALF

A total of 200 µl single cell suspension of washed and resuspended BALF was centrifuged using a Cytospin (Hettich, Germany) at 1500 r/min for 10 minutes and then stained with Wright-Giemsa solution. And 300–400 cells were counted in at least twenty different fields based on standard morphological criteria and staining properties at original magnification ×1000. Cells with epithelial cell or fibroblast morphology were excluded to obtain total leukocyte counts and the proportion of macrophages/monocytes, lymphocytes, eosinophils and neutrophils was calculated.

Histological and immunohistochemical examination

Left lungs were fixed with 4% paraformaldehyde

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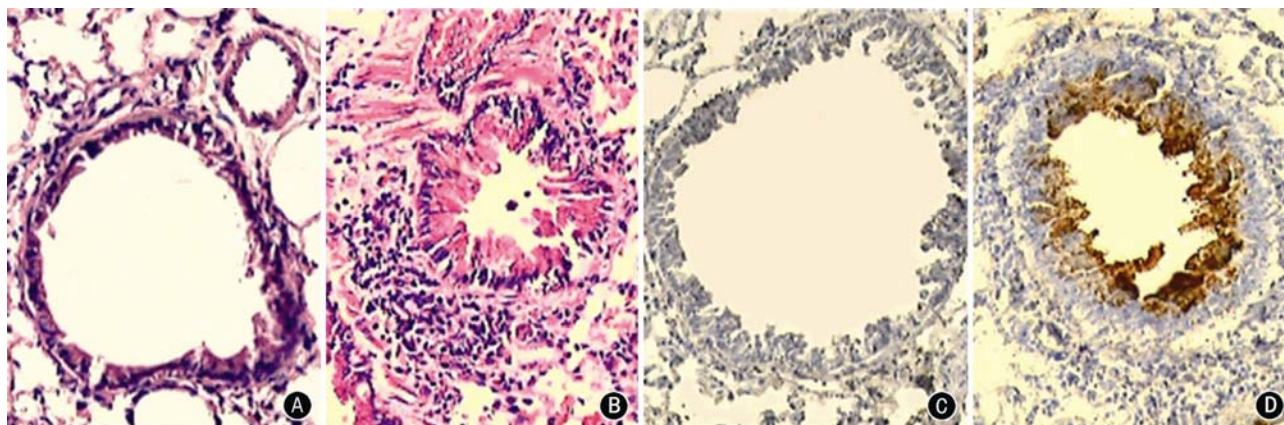
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Table 1. Absolute numbers of total cells, macrophages/monocytes, eosinophils, lymphocytes and neutrophils in BALF of animals ($\times 10^4$, mean $\pm$ SD)

Groups	n	Total cells	Macrophages/monocytes	Eosinophils	Lymphocytes	Neutrophils
Control group	8	11.67 $\pm$ 1.97	10.98 $\pm$ 2.02	0.01 $\pm$ 0.12	0.64 $\pm$ 0.12	0 $\pm$ 0
OVA group	8	53.46 $\pm$ 4.08*	23.31 $\pm$ 3.52*	15.37 $\pm$ 1.06*	14.13 $\pm$ 1.20*	0.65 $\pm$ 0.35*

*Compared with the control group, $P < 0.05$.**Figure 1.** HE and immunohistochemical staining of lung tissues. **A, B:** Control group and OVA group with HE staining respectively (original magnification $\times 200$). Abundant inflammatory cells can be readily noticed on OVA group. **C, D:** Immunohistochemical staining for mclca3 in control group and OVA group respectively. Positive expression of mclca3 was stained brown (original magnification $\times 200$).

(Sigma, USA) and embedded in paraffin. Five- μ m sections were stained with hematoxylin and eosin for histological analysis. Cellular localization of mclca3 positive cells was studied by immunohistochemistry with S-P method. Phosphate buffered saline was used to replace polyclonal rabbit anti-mouse mclca3 antibody (Abcam, UK; 1:800 diluted) to serve as negative control.

Reverse transcription-polymerase chain reaction

Total RNA was isolated from lung specimens with Trizol (Invitrogen, USA) according to the manufacturer's protocol. First-strand complementary cDNAs were synthesized from 1 μ g total RNA with a cDNA extraction kit (Toyobo, Japan). The polymerase chain reaction (PCR) was performed with 1 μ l each of the forward and reverse primers, 1 μ l of cDNA, 10 μ l of 2 $\times$ EasTaq PCR SuperMix (TransGen Biotech, Beijing, China), and distilled water was added to bring the reaction volume to 20 μ l. The primer sequences for mouse mclca3 were 5'-CACAACCACTAAGGTGGCCT-3' (sense) and 5'-AGGTGTTGAAGTGGTCCCTG-3' (antisense), and those for β -actin were 5'-TCCTGTGGCATCCATG-AACT-3' (sense) and 5'-GAAGCACTTGCGGTGCA-CGAT-3' (antisense). All primers were synthesized by Shanghai Bio-engineering (China). The amplification reaction of mclca3 was carried out as follows: 94 $^{\circ}$ C for 5 minutes followed by 31 cycles at 94 $^{\circ}$ C for 30 seconds, 53 $^{\circ}$ C for 45 seconds, and 72 $^{\circ}$ C for 45 seconds, followed by the final step at 72 $^{\circ}$ C for 10 minutes. And β -actin was performed as follows: 94 $^{\circ}$ C for 5 minutes followed by 24 cycles at 94 $^{\circ}$ C for 30 seconds, 58 $^{\circ}$ C for 40 seconds, and 72 $^{\circ}$ C for 30 seconds, followed by the final step at 72 $^{\circ}$ C for 10 minutes. The expected size for mouse mclca3 was 170 bp, and for β -actin was 315 bp. The amplified products were electrophoresed in 2% agarose agar and

quantified by gel image analysis system. The level of mouse mclca3 gene expression was semiquantitative by calculating the ratio of densitometric readings of the band intensity for mouse mclca3 and β -actin from the same cDNA sample.

Measurement of BALF chemokines by ELISA

Levels of chemokines were determined by commercial ELISA kits in accordance with the manufacturer's instructions. ELISA kits for detection of CCL5/RANTES and CCL17/TARC in supernatants were obtained from Boster (Wuhan, China) and Westang (Shanghai, China), respectively. The minimum detectable concentration of each kit was estimated to be lower than 3 pg/ml and 15 pg/ml.

Statistical analysis

Data are expressed as the mean $\pm$ standard deviation (SD). Independent samples *t* test and correlation analysis were performed with SPSS version 15.0 for windows. A $P < 0.05$ was considered statistically significant.

This study adhered to the ethics requirements for the care and use of laboratory animals which had been approved by Hubei Province Animal Caring Committee.

RESULTS

Significantly increased levels of inflammatory cell infiltrates were found in OVA group compared with control group both in lung tissues and in BALF, especially lymphocytes and eosinophils in their BALF (Figure 1 and Table 1).

Mclca3 protein expression was not detected in control

group while strong immunoreactivity was detected in OVA group, which was strictly localized to the airway epithelium (Figure 1).

Mclca3 mRNA was positively expressed in OVA group, but was negatively expressed in control group (Figure 2). The mclca3 mRNA level determined by ratio of mclca3 and β -actin was 1.68 ± 0.72 in OVA group compared with 0 ± 0 in control group ($P < 0.05$).

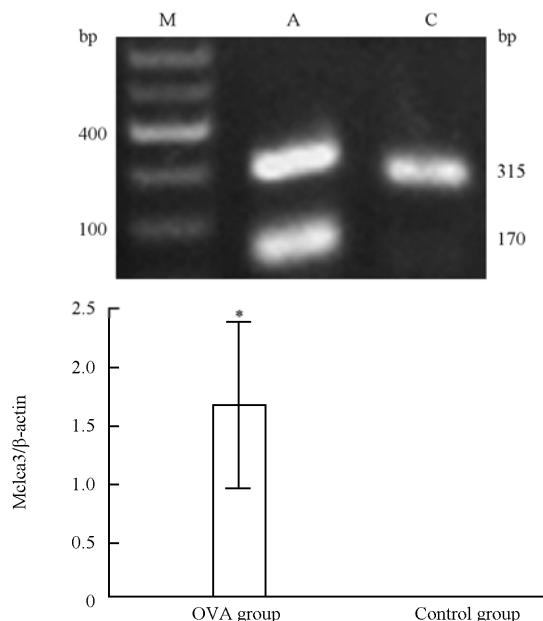


Figure 2. Mclca3 mRNA expression in lung tissues of mice in the 2 groups ($n=8$ of each group). M: Marker; C: control group; A: OVA group. mclca3: 170 bp, 32 cycles; β -actin: 315 bp, 25 cycles. * Compared with the control group, $P < 0.05$.

Levels of the chemokines in BALF in OVA group were significantly increased compared with control group (Table 2).

Table 2. Contents of chemokine proteins in BALF of each group (mean $\pm$ SD)

Groups	<i>n</i>	RANTES/CCL5 (pg/ml)	CCL17/TARC (pg/ml)
Control group	8	9.3925 $\pm$ 1.4960	85.1532 $\pm$ 10.2289
OVA group	8	17.6678 $\pm$ 3.4886*	124.2813 $\pm$ 18.5373*

*Compared with the control group, $P < 0.05$.

In OVA group, mclca3 mRNA levels in lung tissue were positively correlated with CCL17/TARC protein ($r=0.747$, $P < 0.05$) or RANTES/CCL5 protein ($r=0.727$, $P < 0.05$) in BALF.

DISCUSSION

As an asthma-related gene confirmed by many researchers, the role of mclca3 in asthma is elusive. Previous reports about mclca3 were mainly focused on its role in mediating the overproduction of mucus in airway.⁶⁻⁸ By contrast, few studies about its effects on immune or inflammatory response can be found. In this study we sought to investigate the correlation between

mclca3 and two chemokines secreted by airway epithelial cells and hoped to find some hints about the action of mclca3 with airway inflammation in asthma.

In our research, strong up-regulation of the mclca3 mRNA and protein was observed by RT-PCR and immunohistochemistry in OVA group compared with control group. In agreement with previous reports,⁷ we found that the mclca3 protein was exclusively detected in the airway epithelium. This striking change indicated the potential connection between mclca3 and airway epithelium in OVA-challenged mice. Meanwhile, abundant inflammatory cells observed in lung tissue sections were a reflection of the development of perivascular and peribronchial infiltrates. And mice in OVA group had a significant greater number of inflammatory cells especially lymphocytes and eosinophils in their BALF compared with control mice which agreed with previous report.⁹ Recently, the pathogenic role of airway epithelial cells in airway allergic inflammation has become clear since these cells have been identified as a potent source of eosinophil-specific chemokines such as eotaxin, RANTES/CCL5, MCP-4 and Th2-type chemokine TARC/CCL17.¹¹ Studies in asthma have shown that CCL11/eotaxin and CCL5/RANTES are potent chemoattractants for eosinophils, whereas CCL17/TARC and CCL22/MDC play a key role in the recruitment of Th2 cells into the site of allergic inflammation.^{12,13} CCL17/TARC is a CC chemokine that binds specifically to CCR4 to induce chemotaxis of CD4-Th2 cells. Besides CCR4, Th2 cells typically express the chemokine receptors CCR8 and CCR3, however, after repeated challenge such as in our model, CCR4-mediated Th2 recruitment predominates.^{14,15} Therefore, we focused on TARC/CCL17 and RANTES/CCL5. Our data suggest the levels of CCL17 and RANTES are both increased in BALF in OVA group compared with control group. Furthermore, the positive correlation between mclca3 and CCL17 (0.747, $P < 0.05$), and between mclca3 and RANTES (0.727, $P < 0.05$) in OVA group implicates the over expression of mclca3 in asthmatic mice, which may modulate the recruit of inflammatory cells by regulating the function of chemokines derived from airway epithelial cells.

However, our study has several limitations. First, as a simple block design, our experiment could not confirm the steps in the causal chain from mclca3 to inflammatory cells infiltration and to airway inflammation. Thus, genetic tools to repress mclca3 should be used as a control group. Second, the spectrum of chemokines/chemokine receptors expressed at the inflammatory site is complicated.¹² Only two kinds of chemokines could not explain all. With further study, more interrelated chemokines/chemokine receptors should be chosen and neutralizing antibody of some chemokines/chemokine receptors should be applied. Finally, although airway epithelial cells are important source of TARC/CCL17 and

RANTES/CCL5 in asthma, other cells such as dendritic cells, monocyte/macrophagea can also produce them. To help resolve this dilemma, we are trying to construct an airway epithelial cell targeted vector.

In summary, the current study provides preliminary evidence that over expression of mclca3 in epithelial cells may play an important role in inflammatory immunoreaction in asthma. Our report may provide a new insight into the effect of mclca3 in the pathogenesis and treatment of asthma.

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