

Original article

Murine calcium-activated chloride channel family member 3 induces asthmatic airway inflammation independently of allergen exposure

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Keywords: murine calcium-activated chloride channel family member 3; asthma; inflammation

Background Expression of murine calcium-activated chloride channel family member 3 (mCLCA3) has been reported to be increased in the airway epithelium of asthmatic mice challenged with ovalbumin (OVA). However, its role in asthmatic airway inflammation under no OVA exposure has not yet been clarified.

Methods mCLCA3 plasmids were transfected into the airways of normal BALB/c mice. mCLCA3 expression and airway inflammation in mouse lung tissue were evaluated. Cell differentials and cytokines in bronchoalveolar lavage fluid (BALF) were analyzed. The expression of mCLCA3 protein and mucus protein mucin-5 subtype AC (MUC5AC) were analyzed by Western blotting. The mRNA levels of mCLCA3, MUC5AC and interleukin-13 (IL-13) were determined quantitatively.

Results mCLCA3 expression was not detected in the control group while strong immunoreactivity was detected in the OVA and mCLCA3 plasmid groups, and was strictly localized to the airway epithelium. The numbers of inflammatory cells in lung tissue and BALF were increased in both mCLCA3 plasmid and OVA groups. The protein and mRNA levels of mCLCA3 and MUC5AC in the lung tissue were significantly increased in the mCLCA3 plasmid and OVA groups compared to the control group. The level of IL-13, but not IL-4, IL-5, IFN- γ , CCL2, CCL5 or CCL11, was significantly increased compared with control group in BALF in the mCLCA3 plasmid and OVA groups. The level of IL-13 in the BALF in the mCLCA3 plasmid group was much higher than that in the OVA group ($P < 0.05$). The level of mCLCA3 mRNA in lung tissue was positively correlated with the levels of MUC5AC mRNA in lung tissue, IL-13 mRNA in lung tissue, the number of eosinophils in BALF, and the content of IL-13 protein in BALF. The level of IL-13 mRNA in lung tissue was positively correlated with the number of eosinophils in BALF and the level of MUC5AC mRNA in lung tissue.

Conclusion These findings suggest that increased expression of a single-gene, mCLCA3, could simulate an asthma attack, and its mechanism may involve mCLCA3 overexpression up-regulating IL-13 expression.

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Asthma is a complex inflammatory disorder of the airways associated with the intricate interplay of genetic and environmental factors.¹ However, it remains controversial as to whether genetic or environmental factors plays the major role. Physiologic studies and genomic analysis have shown that murine calcium-activated chloride channel family member 3 (mCLCA3) is an asthma-related gene. mCLCA3 is one of the CLCA family members, which play important roles in diverse processes including cell volume regulation, cellular excitability, cell cycle, and muscle tone. It has been reported that mCLCA3 is induced in the airway epithelium of asthmatic mice challenged with Ovalbumin (OVA).² However, its role in asthmatic airway inflammation without OVA exposure has not yet been clarified. In this study, a suspension of pcDNA3.1-mCLCA3 plasmid and transfection reagent ExGen500 was installed intranasally into the lungs of normal mice. We assayed the expression of MUC5AC and markers of airway inflammation to investigate whether increased production from a single-gene, mCLCA3, could simulate an asthma attack and asthmatic inflammation and we explored the possible mechanisms.

METHODS

Experimental animals

Thirty-six eight-week-old specified-pathogen 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 three groups: control group (unsensitized mice treated with a control plasmid and challenged with PBS, $n=10$); OVA group (OVA-sensitized on day 0 and day 14, OVA-challenged from day 21 to day 23, $n=10$); mCLCA3 plasmid group (pcDNA3.1-mCLCA3 plasmids were installed intranasally into unsensitized mice 24 hours before each of the three PBS challenges, $n=10$). The method of plasmid transfection into airways was established as previously described.³ Twenty-four hours after the last challenge, the mice were

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sacrificed and the bronchoalveolar lavage fluid (BALF) and lung specimens were collected.

Bronchoalveolar lavage

Whole lung lavage was performed for each mouse; 0.7 ml of room temperature sterile saline was twice infused into the lungs to total lung capacity 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 cells were counted in at least twenty different fields based on standard morphological criteria and staining properties at magnification $\times 1000$. Cells with epithelial cell or fibroblast morphology were excluded to obtain total leukocyte counts and the proportion of macrophages/monocytes, lymphocytes, eosinophils, or neutrophils were calculated.

Histological and immunohistochemical examination

Left lungs were fixed with 4% paraformaldehyde (Sigma, USA) and embedded in paraffin. Five- μm sections were stained with hematoxylin and eosin for histological analysis. Cellular localization of mCLCA3 and MUC5AC positive cells was determined by immunohistochemistry with the S-P method. Phosphate buffered saline was used to replace polyclonal rabbit anti-mouse mCLCA3 antibody (Abcam, UK; 1:800) and rabbit polyclonal anti-MUC5AC antibody (Boster, Wuhan, China; 1:500) to serve as negative controls. The S-P kit was obtained from Boster (Wuhan).

Detection of protein content of mCLCA3 and MUC5AC in lung tissues with Western blotting

Lung tissue was homogenized in Lysis 13 (0.01 mol/L Tris-HCl pH 7.4, 100 mmol/L NaCl, 10 mmol/L EDTA, 0.1 mmol/L PMSF, 5 $\mu\text{g}/\text{ml}$ aprotinin). Samples were centrifuged at $600 \times g$ for five minutes, and supernatant protein (75 mg per lane) was separated by electrophoresis on a 12% sodium dodecyl sulfate-polyacrylamide gel, and transferred to nitrocellulose membranes. After blocking for two hours at room temperature, the membranes were incubated overnight with the primary antibodies (rabbit polyclonal anti-mCLCA3 antibody 1:200 or rabbit polyclonal anti-MUC5AC antibody 1:500 and rabbit polyclonal anti- β -actin 1:1000). The anti-mouse mCLCA3 antibody was obtained from Abcam (Abcam, UK) and other antibodies were obtained from Boster (Wuhan). Finally, the membranes were incubated with horseradish peroxidase conjugated IgG (1:4000) for two hours at room temperature and visualized using an ECL kit (Boster, Wuhan). The relative amount of immunoreactive mCLCA3 and MUC5AC present in each sample was quantitated by

densitometry (AMBIS Radioanalytic and Visual Imaging System, Ambis Inc.). The densitometric data from each blot was normalized to the control condition with the control value set equal to one for each experiment. All tests were repeated three times.

Measurement of BALF cytokines and chemokines by ELISA

Levels of cytokines and chemokines were determined by commercial ELISA kits in accordance with the manufacturer's instructions. ELISA kits for detection of IL-4, IL-5, IL-13, IFN- γ , CCL2, CCL5, and CCL11 in supernatants were obtained from Boster (Wuhan). The minimum detectable concentration for each factor was 3 pg/ml.

Isolation of total RNA and real-time quantitative PCR

The total RNA was extracted from 30 lung tissue samples from mice using the Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. The RNA samples were quantified by A260. RNA was reverse transcribed to cDNA using an RT-PCR kit (TaKaRa, Japan) according to the kit protocol. The cDNA was analyzed immediately or stored at -20°C . Quantitative-PCR amplification was performed by the ABI 7900HT Fast Real-Time PCR System (ABI Company, USA) and the SYBR Green I fluorescent dye method was used to quantify cDNA. Primers used for mCLCA3 detection were: (forward), 5'-ACTAAGGTGGCCTACCTCCAA-3'; (reverse), 5'-GGAGGTGACAGTCAAGGTGAG-3'. Primers used for MUC5AC detection were: (forward), 5'-CCATGCAGAGTCCTCAGAACAA-3'; (reverse), 5'-TTACTGGAAAGGCCCAAGCA-3'. Primers used for IL-13 detection were: (forward), 5'-TGAGCAACATCACACAAGACC-3'; (reverse), 5'-GGCCTTGCGGTTACAGAGG-3'. Primers used for β -actin detection were: (forward), 5'-CTGTCCCTGTATGCCTCTG-3'; (reverse), 5'-ATGTCACGCACGATTTC-3'. All primers were synthesized by Shanghai Bio-engineering Co., Ltd., China. The final volume of the RQ-PCR reaction was 10 μl and included SYBR Premix Ex Taq 5.0 μl , primer (10 $\mu\text{mol}/\text{L}$) 0.2 μl each, 50 $\times$ ROX Reference Dye 0.2 μl , template 1.0 μl , adjust to 10 μl with distilled water. PCR cycling conditions consisted of an initial denaturing step for 10 seconds at 95°C , and then 40 cycles of 5 seconds at 95°C , 30 seconds at 60°C . The β -actin from the same sample was used as an internal control and the relative contents of the copy numbers of the target gene's mRNA were calculated, from which we could determine the gene expression level and the trend of its change. Specificity of each reaction was controlled by melting curve analysis. A negative PCR control containing water instead of cDNA was performed. Real-time PCR was conducted in triplicate in three independent experiments.

Statistical analysis

All data are expressed as mean $\pm$ standard deviation (SD), and comparisons among the three groups were made using

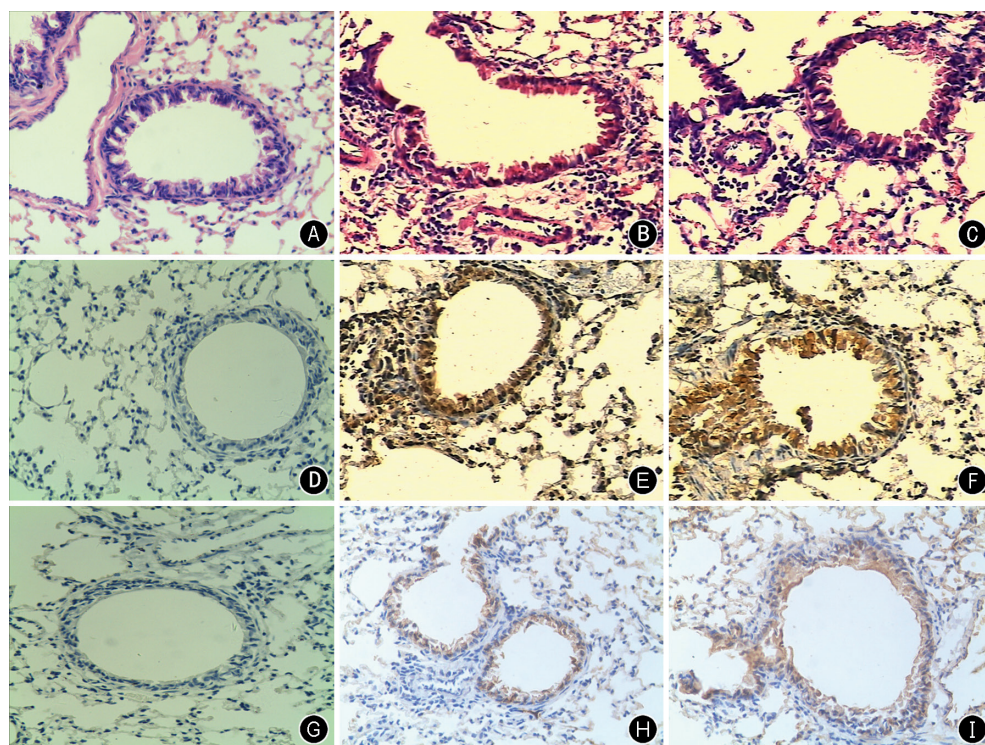


Figure 1. HE and immunohistochemical staining of lung tissues. **A–C:** Control group, OVA group and mCLCA3 plasmid group with HE staining respectively (original magnification $\times 200$). Abundant inflammatory cells can be easily noticed on OVA group and mCLCA3 plasmid group. **D–F:** Immunohistochemical staining for mCLCA3 in control group, OVA group and mCLCA3 plasmid group. Positive expression of mCLCA3 was stained brown (original magnification $\times 200$). **G–I:** Immunohistochemical staining for MUC5AC control group, OVA group and mCLCA3 plasmid group. Positive expression of MUC5AC was stained brown (original magnification $\times 200$).

analysis of variance (ANOVA) followed by the SNK-q test. Correlation analyses were assessed with the Pearson test. Statistical analysis was performed using the SPSS 15.0 software package (SPSS Inc.; Chicago, IL, USA) and a P value of less than 0.05 was considered statistically significant.

RESULTS

Symptoms

No asthmatic symptoms were observed in the control

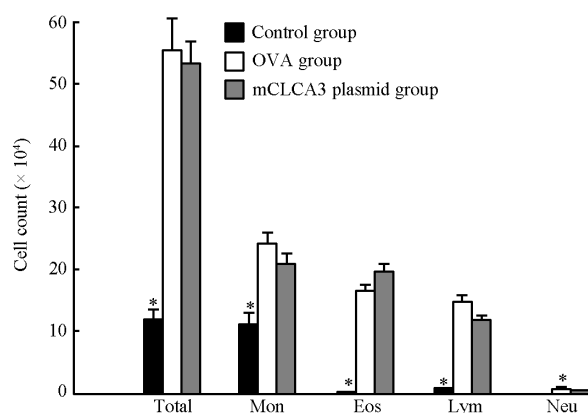


Figure 2. Absolute numbers of total cells, macrophages/monocytes (Mon), eosinophils (Eos), lymphocytes (Lym) and neutrophils (Neu) in BALF of animals. Compared with the OVA group or mCLCA3 plasmid group, $P < 0.05$.

group. The OVA group and mCLCA3 plasmid group showed typical symptoms of asthma such as dyspnoea, tachypnoea, wheezing, muscles of the abdomen ticking, and incontinence. The symptoms of asthma in the mCLCA3 plasmid group were milder than in the OVA group. The observation of asthmatic symptoms were established as previously described.⁴

Airway inflammation

Significantly increased levels of inflammatory cell infiltration were found in the OVA group and mCLCA3 plasmid group compared with the control group in both lung tissues and BALF. There were especially high levels of eosinophils in the mCLCA3 plasmid group (Figures 1 and 2).

Immunohistochemistry for mCLCA3 and MUC5AC

mCLCA3 and MUC5AC expression were not detected in the control group while strong

immunoreactivity were detected in the OVA group and mCLCA3 plasmid group. The mCLCA3 signal was strictly localized to the airway epithelium (Figure 1).

Determination of mCLCA3 and MUC5AC proteins in lung tissues by Western blotting

The target protein mCLCA3 was 90 kD and actin was used as the internal control (43 kD). The level of mCLCA3 was higher in the lung tissues of the mCLCA3 plasmid group and OVA group compared with the control group. The protein level of MUC5AC (213 kD) was also higher in the lung tissues of the mCLCA3 plasmid group and OVA group than control group (Figure 3).

Levels of the cytokines and chemokines in BALF

The level of IL-13 in BALF in the mCLCA3 plasmid group and OVA group were significantly increased compared with the control group ($P < 0.05$). The level of IL-13 in the mCLCA3 plasmid group was significantly higher than in the OVA group ($P < 0.05$). The levels of IL-4, IL-5, CCL2, CCL5 and CCL11 in BALF were significantly increased in the OVA group compared with the control group or mCLCA3 plasmid group. IFN- γ in BALF was significantly lower in the OVA group compared with the control group or mCLCA3 plasmid group (Figure 4).

Expression level of mCLCA3, MUC5AC and IL-13 mRNA in the lung tissues of mice

mCLCA3, MUC5AC and IL-13 mRNA were positively

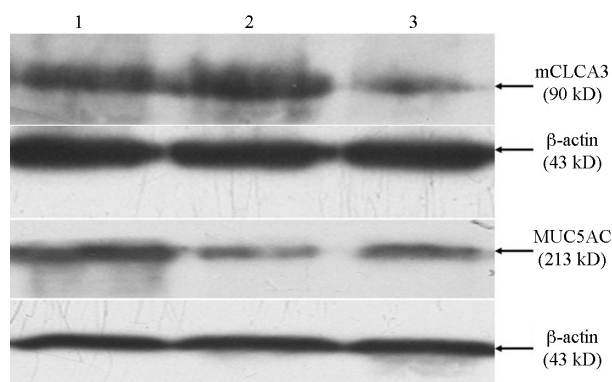


Figure 3. The contents of mCLCA3 and MUC5AC proteins in the lung tissues shown by Western blotting. 1: mCLCA3 plasmid group; 2: OVA group; 3: control group.

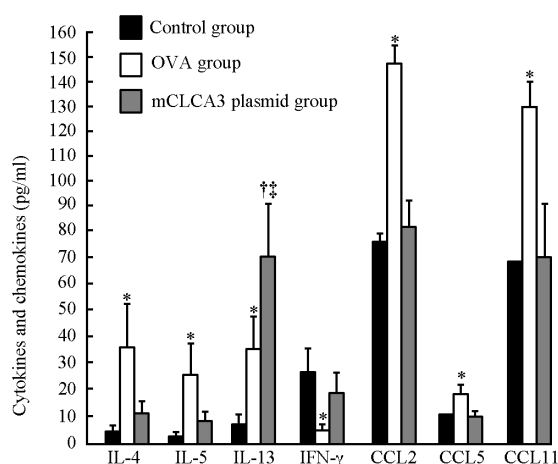


Figure 4. Contents of chemokine proteins in BALF of each group (mean±SD). *Compared with the control group, $P < 0.05$; †Compared with the OVA group, $P < 0.05$.

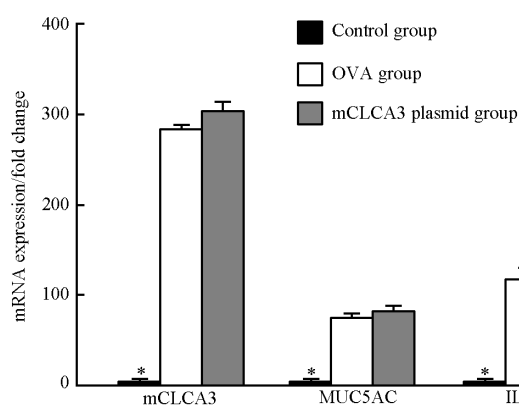


Figure 5. Levels of mCLCA3, MUC5AC and IL-13 mRNA in lung tissues in each group (mean±SD). *Compared with OVA group or mCLCA3 plasmid group, $P < 0.05$; †Compared with the OVA group, $P < 0.05$.

expressed in the mCLCA3 plasmid group and OVA group. The levels of mCLCA3 and MUC5AC in the mCLCA3 plasmid group were slightly higher than in the OVA group ($P > 0.05$), while the level of IL-13 in the mCLCA3 plasmid group was significantly higher than in the OVA group ($P < 0.05$) (Figure 5).

Correlation analysis

The level of mCLCA3 mRNA in lung tissue was positively correlated with the levels of MUC5AC ($r=0.602$, $P < 0.05$) and IL-13 ($r=0.856$, $P < 0.05$) mRNA in lung tissue, the content of IL-13 protein in BALF ($r=0.917$, $P < 0.05$) and the number of eosinophils in BALF ($r=0.587$, $P < 0.05$). The level of IL-13 mRNA in lung tissue was positively correlated with the number of eosinophils in BALF ($r=0.515$, $P < 0.05$) and the levels of MUC5AC mRNA in lung tissue ($r=0.556$, $P < 0.05$).

DISCUSSION

The overexpression of the mCLCA3 gene by transfecting exogenous mCLCA3 genes into airway epithelial in a murine asthma model has been reported; with exacerbated AHR, goblet cell hyperplasia, mucus overproduction, and eosinophil infiltration.² The mCLCA3 gene in mice corresponds to the CLCA1 gene in humans, which can regulate calcium activated chloride conductance. hCLCA1 has been shown to induce mucin gene expression (MUC5AC) in the human mucocoepermoid cell line NCI-H292 and its expression was demonstrated to be increased in patients with asthma.^{5,6} Our analysis of cytokine production and cell differentials in BALF in the OVA-challenged asthmatic mouse model with airway inflammation has also been previously reported.⁷ However, all of these reports have been investigated only in murine asthma models with a exposure to OVA allergens, which means that there were two variables, the expression of mCLCA3 and sensitization with OVA. It was not determined which one was the initiating factor, the abnormal overproduction of an individual gene product or the external environmental stimuli. In order to elucidate the role of an individual gene (mCLCA3) in asthmatic airway inflammation and mucus overproduction, we designed a single gene overexpression model, and then focused on airway inflammation, mucus protein secretion and possible mechanisms.

In our study, the mCLCA3 plasmid group showed typical symptoms of asthma such as dysphoria, tachypnoea, wheezing, muscles of the abdomen ticking, and incontinence. Strong up-regulation of the mCLCA3 mRNA and protein were observed by real-time polymerase chain reaction (PCR), Western blotting and immunohistochemistry in the mCLCA3 plasmid group compared with the control group. The level of mCLCA3 in the mCLCA3 plasmid group was a little higher than that in the OVA group. We found that the mCLCA3 protein was exclusively detected in the airway epithelium not only in the OVA group, which is in agreement with previous reports, but also in the mCLCA3 plasmid group. This striking change indicated a potential association between mCLCA3 and airway epithelium. Meanwhile, abundant inflammatory cells observed in lung tissue sections were a reflection of the seriousness of perivascular and peribronchial infiltrations. The mice in the OVA group had greater numbers of inflammatory cells in their BALF

than mice in the control group, which is consistent with a previous report.² The mice in mCLCA3 plasmid group had similar numbers of inflammatory cells in their BALF as did the OVA group mice. However, mice in the mCLCA3 plasmid group had higher eosinophil counts in their BALF than mice in the OVA group.

Recently, the pathogenic role of airway epithelial cells in airway allergic inflammation has become clear.⁸ Since these cells have been identified as a potent source of many cytokines and chemokines such as IL-4, IL-5, IL-13, IFN- γ , CCL2/MCP-1, CCL5/RANTES, and CCL11/eotaxin. Studies in asthma have shown that IL-4, IL-5 and IL-13 are Th2-type cytokines, while IFN- γ is a Th1-type cytokine. CCL11/eotaxin and CCL5/RANTES are potent chemoattractants for eosinophils and CCL2/MCP-1 is a key chemoattractant for mononuclear cells. Our data show that the levels of IL-4, IL-5, IL-13, CCL2, CCL5 and CCL11 in BALF were increased in the OVA group compared with the control group. IFN- γ in BALF was decreased in the OVA group compared with the control group. The levels of IL-4, IL-5, IFN- γ , CCL2, CCL5 and CCL11 in BALF were not significantly changed in the mCLCA3 plasmid group compared with the control group; however, the level of IL-13 in BALF was significantly increased in the mCLCA3 plasmid group compared with the control group. Interestingly, the increase of IL-13 in the mCLCA3 plasmid group was much greater than in the OVA group. The level of mCLCA3 mRNA in lung tissue was positively correlated with the level of MUC5AC mRNA, the level of IL-13 mRNA in lung tissue and the content of IL-13 protein in BALF. The content of IL-13 protein in BALF was positively correlated with the number of eosinophils in BALF and the levels of MUC5AC mRNA in lung tissue. The positive correlations between mCLCA3 and MUC5AC, mCLCA3 and IL-13, IL-13 and MUC5AC, as well as IL-13 and eosinophils implies that the overexpression of mCLCA3 might modulate the recruitment of inflammatory cells, especially eosinophils, and also airway epithelial cells might affect the overproduction of the mucus protein MUC5AC by regulating the secretion of IL-13, but not IL-4, IL-5, IL-13, IFN- γ , CCL2, CCL5 and CCL11.

Among Th2 cytokines, IL-13 is considered particularly critical. Local administration of recombinant IL-13 to nonimmunized mice induces the asthma phenotype. The importance of the direct effect of IL-13 on epithelial cells in causing AHR has been demonstrated.⁹⁻¹¹ Airway epithelial cells might produce secreted molecules that alter contraction of airway smooth muscle. A study using murine tracheal rings showed that IL-13 enhanced agonist-induced contractility and calcium signals.¹² The repolarization phase of slow waves in canine airway smooth muscle is mediated by calcium-dependent chloride currents, and slow waves are observed after stimulation with physiologic agonists.^{12,13} Furthermore, *in vivo* blockade of IL-13 markedly inhibits allergen-induced AHR, eosinophilia, and mucus overproduction.¹⁴ In contrast, a few studies found that IL-13 up-regulates MUC2 (another mucin gene) through

CLCA and the epidermal growth factor receptor.^{15,16} These results seem different from ours. Our study indicates that it is possible that different mucin genes might be regulated by different signal pathways, and it is also possible that these pathways could transactivate each other.

In conclusion, we provide evidence that single mCLCA3 gene overexpression is involved in the mucus protein MUC5AC overexpression and the pathogenesis of asthmatic airway inflammation (especially eosinophils infiltration) by inducing IL-13 secretion. Asthma is a complex disorder, and the relevance of the present data to human asthma remains to be fully addressed. It has been reported that IL-13 expression is increased in the airways of patients with asthma and that natural variations in the coding region of IL-13 is an important genetic determinant of susceptibility to allergy. Our findings add to our understanding that the mCLCA3 overexpression could up-regulate IL-13 and induce an asthma phenotype with no OVA exposure. In summary, the current study provides preliminary evidence that overexpression of mCLCA3 in epithelial cells may play an important role in an asthmatic inflammatory reaction. Our report might provide a new insight into the effect of mCLCA3 in the pathogenesis and treatment of asthma.

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