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Safety and Immunogenicity of a DNA Vaccine Encoding Human Calcium-Activated Chloride Channel 1 (hCLCA1) in Asthmatic Mice

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Key Words
Asthma · DNA vaccine · hCLCA1 · mCLCA3

Abstract
Background: Calcium-activated chloride channels (CLCAs) have been found to be preferentially expressed on the secretory epithelium. They may play a pivotal role in mucous overproduction by bronchial goblet cells in asthma. It has been reported that the inhibition of CLCAs with niflumic acid could relieve the symptoms of asthma. However, niflumic acid has serious adverse effects. DNA vaccination is considered to be a promising strategy to treat allergic diseases such as asthma and dust mite allergy. ***Methods:*** We constructed a vaccine encoding human CLCA1 (hCLCA1) and evaluated its effects on promoting antibodies against hCLCA1 and the related preventive function in a mouse model of asthma. ***Results:*** Our results reveal that the induced hCLCA1 antibodies can be detected in the first 2 weeks after immunization with hCLCA1 plasmids (hCLCA1-p) by intramuscular injection and augmented gradually in the following several weeks. The autoantibodies against hCLCA1 induced by the DNA vaccine bound to three segments of the mouse CLCA3 (mCLCA3) protein, including the amino terminal (PepN), the carboxyl terminal (PepC) and the middle of the protein (PepM). In our study, mice immunized with hCLCA1-p developed fewer pathological changes compared with other control groups, including a remarkable reduction in the air pressure-time index of the trachea, the number of eosinophils and mast cells in the bronchoalveolar lavage fluid and the mRNA level of MUC5AC in goblet cells. ***Conclusion:*** Taken together, our results suggest that a DNA vaccine encoding the CLCA protein may have potential as a useful pharmacotherapy for asthma in the future.

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Introduction

Allergic asthma is a common chronic inflammatory disorder in the airways, which is characterized by reversible airflow obstruction, airway inflammation including infiltration of eosinophils, hypersensitivity to various stimuli and mucus hypersecretion. Airway mucus hypersecretion is a cardinal feature of asthma and contributes to the morbidity and mortality of asthma in many patients [1]. The mechanisms underlying mucus hypersecretion have been reported to be related to the expression of Th2 cytokines, such as interleukin (IL)-4, -9 and -13 [2]. These cytokines appear to be directly linked to CLCA overexpression in the asthmatic patient and in mouse models of asthma [3].

L.Q.S. and Y.L. contributed equally to this study.

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Calcium-activated chloride channel (CLCA)) proteins were identified as important molecules on the cell surface, especially cells lining the surface of the secretory epithelium. These channels play an important role in calcium-dependent chloride conductance across epithelial cells and epithelial fluid secretion [4]. In recent years, the CLCA gene family has been extensively studied. The family members have been detected in bovine, human, murine, equine and porcine tissues, and have been shown to be involved in cell-cell adhesion, apoptosis, cell cycle control, tumorigenesis and metastasis [5]. The relationship between CLCAs and asthma was the most commonly investigated aspect in functional studies on CLCAs. HumanCLCA1 (hCLCA1) has been reported to be overexpressed in bronchial allergic asthmatic responses with excessive mucus production [6]. Mucus hypersecretion is responsible for much of the morbidity and mortality associated with airway diseases; it is possible that CLCA-controlled mucus production is especially relevant to clinical aspects of airway diseases, such as asthma [7, 8]. There is significant evidence that the CLCA proteins have a causal role in this condition, suggesting that these proteins may have some interest as targets for pharmacological intervention in allergic asthma [9].

It has been implicated that mouse CLCA3 (mCLCA3) is largely induced in the mouse inflammatory airway and greatly contributes to goblet cell hyperplasia [10, 11]. The significance of research on mCLCA3 is that hCLCA1 is the putative counterpart of mCLCA3 in humans and shares 75% sequence homology with mCLCA3 [12]. It was initially reported that intratracheal administration of an adenovirus expressing the antisense RNA of mCLCA3 RNA into a mouse model of airway hyperreactivity efficiently suppressed the asthmatic phenotype, whereas the overexpression of mCLCA3 in the airway epithelium using an adenoviral vector exacerbated the asthmatic phenotype [10]. Follow-up studies on the mechanisms of CLCA protein function revealed that cytokines, especially IL-13 and IL-9, can augment mCLCA3/hCLCA1 production in asthma; therefore, mCLCA3/hCLCA1 acts as a downstream molecule of the Th2 cytokine network [13]. Studies on the molecular structure of CLCAs have clarified that mCLCA3 and hCLCA1 are both soluble secreted molecules and the precursor, a 120-kDa glycoprotein, can be cleaved into ~85-kDa N-terminal and ~35-kDa C-terminal products, which may serve as subunits of the chloride channel, but are not themselves functional chloride channels.

The outcomes of contemporary pharmacotherapy for severe asthma, for which no specific treatment exists, are still unsatisfying. However, therapies addressing airway mucus hypersecretion have obtained some favorable outcomes [14]. Although specific inhibitors targeting the subtype of CLCA protein are currently not available, DNA vaccination provides an attractive option for protecting an organism against disease through activation of the immune system and antibody production. Following the injection of a genetically engineered DNA molecule, the body will develop an adaptive immune response, including antibodies, helper T cells and cytotoxic T lymphocytes, as well as innate immune responses [15]. Since DNA vaccination has many advantages over traditional vaccines, a variety of DNA vaccines has been developed in human clinical trials against various infectious diseases and cancer, but vaccine therapies against autoimmune diseases and allergy are still in development. Even so, DNA vaccine therapies for allergic asthma have currently mainly focused on allergens. Few of the pathological molecules related to allergic asthma, such as hCLCA1, have been investigated by researchers. Therefore, we proposed that a CLCA DNA vaccine may activate the immune system, thereby inducing antibodies against mCLCA3 protein and resulting in the repression of the mCLCA3-mediated pathological process in asthma. In this study, we demonstrate that an hCLCA1 DNA vaccine prevented mucus hypersecretion and related pathological changes in a murine asthma model through the induction of anti-mCLCA3 antibodies.

Materials and Methods

Animals

Specific pathogen-free male BALB/c mice between 6 and 8 weeks of age were purchased from the Experimental Animal Center of Shaanxi Province, China. Experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the Fourth Military University. The mice were raised with ovalbumin (OVA)-free food and water ad libitum and housed in Macrolon cages in a laminar flow cabinet.

Construction of DNA Plasmid Vaccines

pcDNA3.1 (+)/hCLCA1 and pcDNA/mCLCA3 constructs were gifts from Dr. R.C. Levitt (Genaera Corporation). To establish a new recombinant construct of hCLCA1, the hCLCA cDNA1 fragments in the plasmids were released by digestion with the restriction enzymes *Nhe*I and *Xho*I (Takara Corporation) and identified by DNA sequencing. pcDNA3.1 (+)/hCLCA1 was used as the template for PCR amplification. Meanwhile, new digestion sites were added using the sense primer 5′-CAAGCTTATGGGGCCATTTAAGAGTTC-3′ and the antisense primer 5′-CTAGGCTATTGACAGCTGCA-3′. The PCR product was identified by digestion with *Hind*III and *Xho*I, followed by sequencing to verify the accuracy of the amplified gene. The amplified gene was cloned

into the pSecTag2-B expression vector to construct an hCLCA1 DNA vaccine, which was confirmed by sequencing [13]. The same procedures were applied to the construction of the pSecTag2-B/mCLCA3 plasmid. The primers for full-length mCLCA3 were: sense 5′-GGGAAAGCTGCAGGATGGAATC-3′ and antisense 5′-TATACCGCCCCCTACAGGAAGTCTAA-3′.

Expression of the Recombinant Proteins of Three Extracellular Regions of mCLCA3

The three cDNA segments of the mCLCA3 gene were amplified by PCR using pcDNA3.1 (+)/mCLCA3 as the template. The first segment encoded the amino terminal (PepN; E83~E279), for which the primer pairs were: sense 5′-CGGGATCCATGATTTTGATTCCCGAGAGCTGGA-3′ and antisense 5′-GCGAATTCTTACCCACGTGCTTCGGAGTTTGC-3′. The second encoded the middle section (PepM; N446~I508), for which the primer pairs were: sense 5′-CGGGATCCATGGCTAAAGAGCTTGAGCAGCT-3′ and antisense 5′-CATCCATTGGTTATTCTGGAG-3′. The third was the carboxyl terminal (PepC; S684~Q913), for which the primer pairs were: sense 5′-GGAGGAGTCACTTCAGACAGA-3′ and antisense 5′-TCACCTGCATTTCCCCTAGCC-3′. The cDNA fragments encoding PepN and PepC were cloned into the pRSETB vector, and the segment encoding PepM was cloned into the pGEXT-T1 vector. The three recombinant proteins were produced by transforming *Escherichia coli* DE3 cells using three plasmids of recombinant mCLCA3 and IPTG induction for 4 h before harvesting the bacteria.

Purification of the Recombinant Proteins of Three Extracellular Regions of mCLCA3

The recombinant proteins were purified using a previously described method with minor modifications [16]. The bacterial precipitate was resuspended in inclusion body wash buffer [1 mol/l Tris-HCl (pH 0.9), 0.5 mol/l EDTA, 5 mol/l NaCl, glycerol, 0.3 mol/l DTT], and PMSF was added to achieve a final concentration of 10 mmol/l, followed by ultrasonic degradation and centrifugation (10 min at 12,000 *g* at 4°C). The precipitate was dissolved in washing buffer of inclusion body and sodium deoxycholate for 10 min and then centrifuged for 10 min at 12,000 *g* at 4°C. After washing, the inclusion bodies were dissolved by washing buffer of inclusion body and SKL for 50 min, centrifuged (10 min at 12,000 *g* at 4°C). The supernatant was dialyzed in washing buffer of inclusion body for 24 h. To evaluate the recombinant protein, the bacterial lysates were resolved via 10% SDS-PAGE, followed by blotting and Coomassie blue staining.

Determination of Antibody Titers by ELISA

Sera from immunized mice were collected and the titers of antibodies against PepN, PepM and PepC were examined by indirect ELISA. Briefly, 96-well microtiter plates were coated with 5 mg/l of the recombinant PepN, PepM or PepC proteins, which were diluted in sodium carbonate buffer at 4°C overnight. Anti-goat IgG horseradish peroxidase was used as the secondary antibody. The plates were washed, and detection was performed using sodium citrate buffer, containing 1 mg/ml 3,5,5-tetramethylbenzidine and 0.03% H_2O_2. The reaction was stopped by adding 50 μl of 0.25% hydrofluoric acid to each well. The plates were read at an absorbance of 630 nm. All experiments included 12 mice per group and were repeated at least three times.

Western Blot Analysis

A total of 8 μl of bacterial lysate was fractionated by 15% SDS-PAGE and transferred to a nitrocellulose membrane. The efficiency of electrotransfer was verified by Ponceau staining. The membrane was blocked in 5% skim milk for 30 min and then blotted with immunized murine serum at a working dilution of 1:50 at 4°C overnight. A horseradish peroxidase-linked goat anti-mouse antibody (Boster Corporation; diluted 1:1,000) was used as the secondary antibody. Finally, the blots were detected using enhanced chemiluminescence reagents. All experiments included 12 mice per group and were repeated at least three times.

Murine Asthma Model

Mice were injected with 50 μl of 50% bupivacaine into the bilateral muscle of the posterior thigh prior to immunization; 24 h later, the mice were immunized at the same site with 100 μg of the hCLCA1 DNA vaccine, which was dissolved in 50 μl of PBS. Successive immunization was performed three times once every 2 weeks and blood samples were collected at the same time by cutting off a small piece of the tail. On the day after the last immunization, the mice were sensitized by an intraperitoneal injection of OVA (20 μg) along with 2 mg of aluminum hydroxide. One week after the sensitizing injection, mice were injected again with 10 μg of OVA. Two weeks after the first sensitizing injection, the mice were daily challenged for 30 min with an aerosol of 5% (w/v) OVA for 5 consecutive days. Sham-challenged mice inhaled an aerosol of PBS alone using the same protocol [17, 18]. All experiments included 12 mice per group and were repeated at least three times.

Airway Hyperresponsiveness

Airway hyperresponsiveness was evaluated on the day after the last challenge, as described in the literature [19]. Briefly, mice were anesthetized with pentobarbital sodium, and the trachea and caudal vein were cannulated. Then animals were artificially ventilated by a breathing machine with a tidal volume of 8 ml/kg at a rate of 60 breaths/min. Airway pressure was measured through a minute pressure transducer. The mice were injected a range of methacholine (Sigma Corporation) doses (from 0.1 to 1.5 mg/kg i.v.) at 10-min intervals using a syringe. Airway responsiveness to methacholine, represented by the airway pressure-time index (APTI), was assessed after intravenous injection of mice. All experiments included 12 mice per group and were repeated at least three times.

RT-PCR for the Detection of MUC5AC

Total RNA was extracted from frozen lung tissue using TRIzol reagent (Invitrogen) following the manufacturer's protocol. RT-PCR was performed by reverse transcription using 1 μg of total RNA and the appropriate primers. Custom-designed specific primer pairs for MUC5AC were: sense 5′-GTGGAACCACG ATGA-3′ and antisense 5′-TCAGCACATAGCTGCAGTCG-3′. A cDNA fragment of GAPDH was amplified as the control [12, 20].

Statistical Analyses

Data are presented as means ± SD. One-way analysis of variance was used to compare values among multiple groups followed by unpaired two-tailed Student's t tests. Differences were considered statistically significant at $p < 0.05$. All experiments were repeated at least three times.

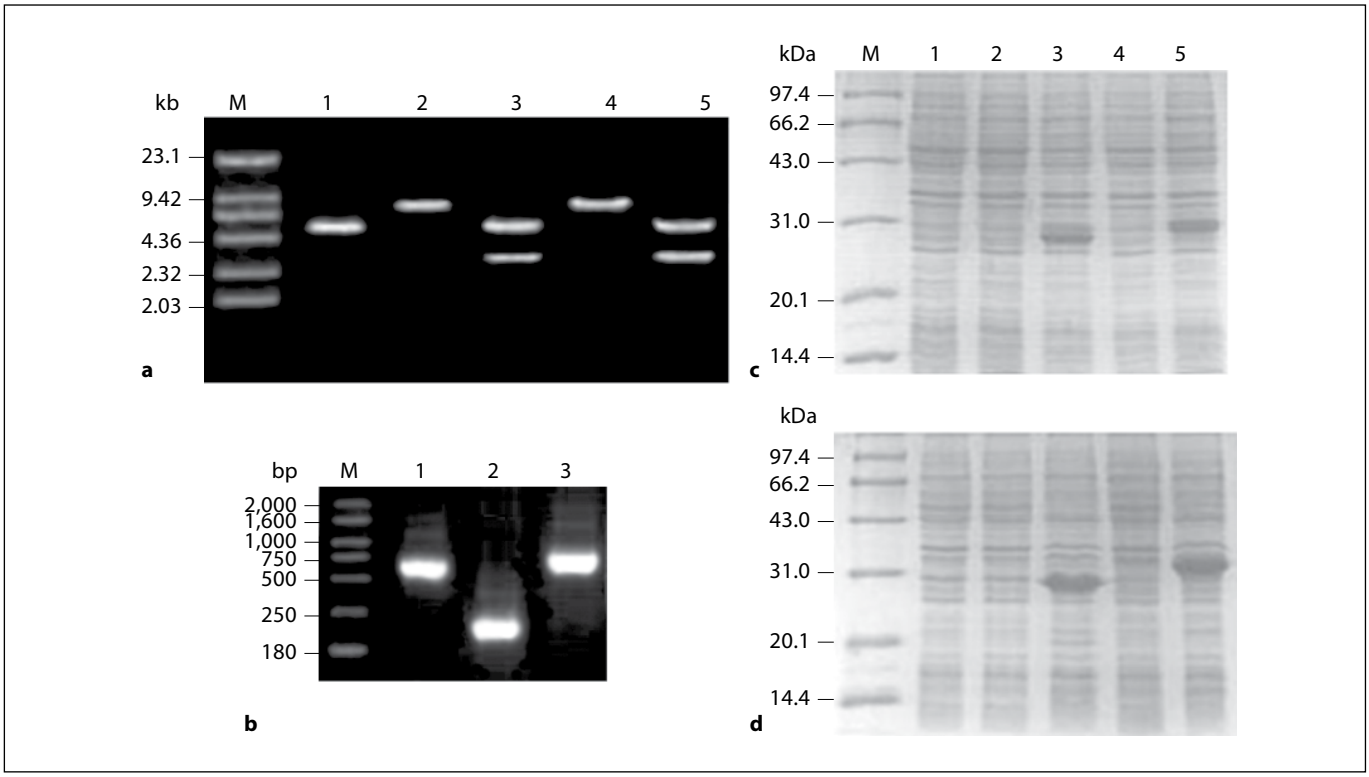


Fig. 1. Identification of the hCLCA1-DNA vaccine and mCLCA3 peptides. **a** Agarose gel electrophoresis for verification of hCLCA1-p and mCLCA3-p. M = DNA marker; 1 = the hollow vector of pSecTag-2B; 2 = hCLCA1-p; 3 = hCLCA1-p digested with *Hind*III and *Xho*I; 4 = mCLCA3-p; 5 = mCLCA3-p digested with *Hind*III and *Xho*I. **b** Identification of PCR products. M = DNA marker; 1 = PepN cDNA; 2 = PepM cDNA; 3 = PepC cDNA. **c**, **d** Identification of the recombinant mCLCA3 peptides. **c** M = Protein marker; 1 = lysates of normal DE3 cells; 2, 3 = DE3 cells transformed by PepN-p without or with induction of IPTG; 4, 5 = DE3 cells transformed by PepC-p without or with induction of IPTG; **d** M = Protein marker; 1 = lysates of normal DE3 cells; 2, 3 = DE3 cells transformed by the vector of pGEXT-T1 without or with induction of IPTG; 4, 5 = DE3 cells transformed by PepM-p without or with induction of IPTG.

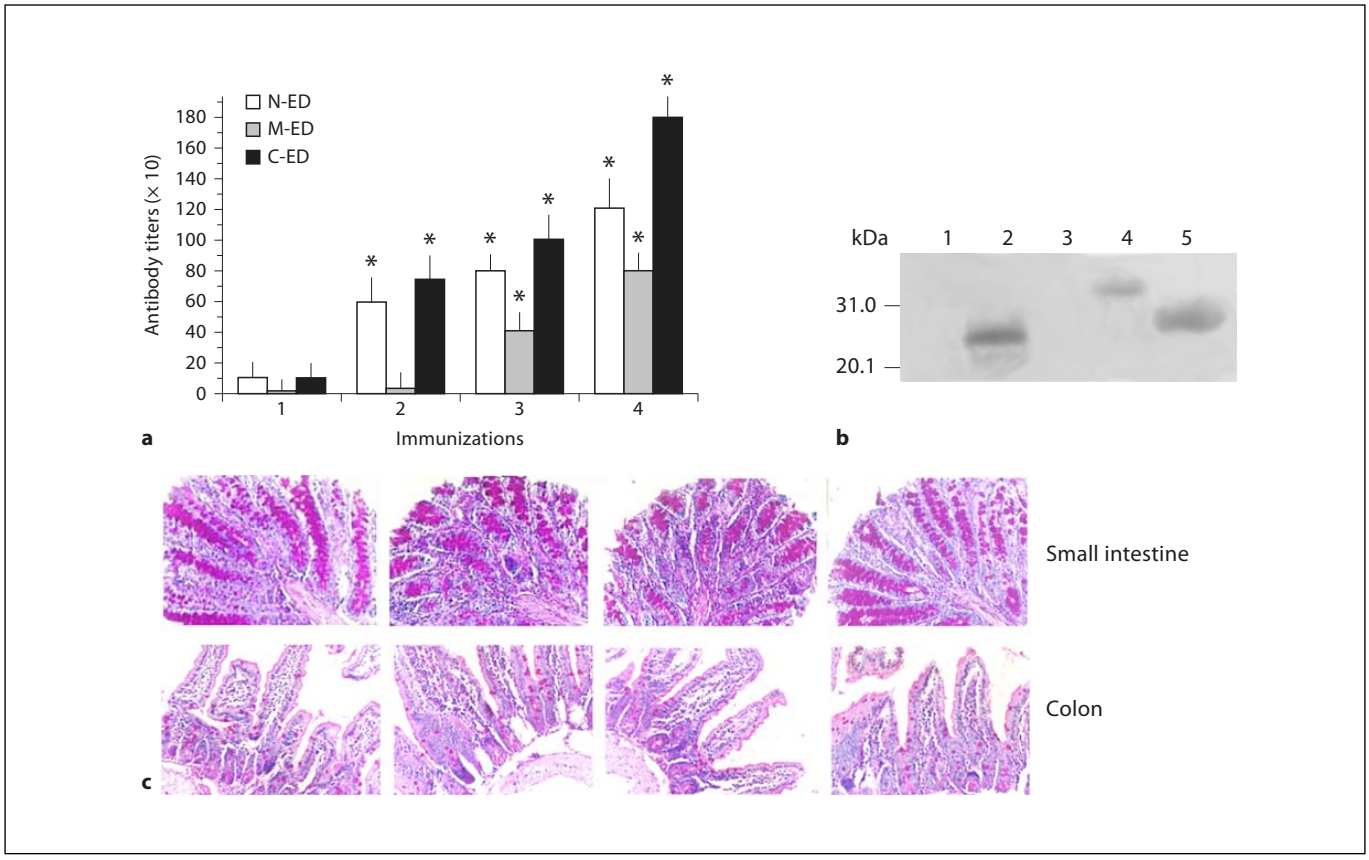
Results

Characteristics of the hCLCA1-DNA Vaccine

The pSecTag2-B/hCLCA1 and pSecTag2-B/mCLCA3 plasmids were digested with restriction enzymes and sequenced as described above. The plasmids were verified by agarose gel electrophoresis. The recombinant plasmids released a fragment of 2.7 kb, as shown in figure 1a. Alignment using BLAST (Basic Local Alignment Search Tool) confirmed the sequencing results were identical to AA541829 and AA508854 (GenBank).

Alignment analysis of the protein sequence of mCLCA3 and hCLCA1 found three segments that were different in their sequences at the N-terminal, middle and C-terminal regions, which had the potential to be exogenously antigenic. We cloned these three segments of mCLCA3 individually. The cDNA sizes of PepN, PepC and PepM were 591, 687 and 186 bp, respectively. The PCR products for cloning were identified by agarose gel electrophoresis (fig. 1b). A prokaryotic expression system was used to produce the recombinant peptides. The molecular weights of the PepN and PepC peptides were around 25.21 and 29.31 kDa, respectively, and an additional 6 histidines were added to PepN and PepC in *E. coli* DE3 cells. The cDNA of PepM was cloned into the pGEXT-T1 vector and fused to the GST protein (26 kDa). SDS-PAGE was carried out to confirm the production of the three peptides (fig. 1c, d).

To estimate the efficacy of immunization with the hCLCA1 DNA vaccine, the antibody titers in the serum of immunized mice were examined by ELISA, in which the serum was used as the primary antibody. The results showed that antibodies to PepN, PepM and PepC were detectable in the serum 2 weeks after the 1st dose challenge



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Fig. 2. Characterization of the hCLCA1-DNA vaccine. **a** Antibody titers for the three mCLCA3-ED in the serum of mice immunized with hCLCA1-p at different time points. * $p < 0.01$ vs. 1st immunization. **b** Western blot for polyclonal autoantibodies in murine serum 6 weeks after the first challenge. 1 = Normal DE3 cells; 2 = DE3 cells transformed by PepN-p; 3 = DE3 cells transformed by the vector of pGEXT-T1; 4 = DE3 cells transformed by PepM-p; 5 = DE3 cells transformed by PepC-p. **c** Detection of mucus secretion by goblet cells in the colon and small intestine (×100) by PAS staining. ED = Extracellular domain.

of hCLCA1 plasmids (hCLCA1-p) and gradually increased as immunization continued (fig. 2a). However, the titer of the PepM antibody was lower than that of antibodies to PepN and PepC. Antibody production had doubled after the 4th immunization compared with the 2nd immunization. However, the other four groups were all negative and did not produce any antibody (data not shown). To further confirm the specificity of the antibodies to the 3 peptides, serum was collected after the last immunization and antibodies were detected by Western blot analysis, as described in the Materials and Methods (fig. 2b).

During the immunization process, no abnormalities were observed in the activity, diet or growth conditions of the immunized mice, and no respiratory side effects or deaths occurred. The small intestine and colon of the immunized mice were removed and paraffin sections were stained with periodic acid-Schiff (PAS). The results demonstrate no apparent damage or loss of mucus-secreting goblet cells in hCLCA1-p-immunized mice compared with control mice, suggesting that the DNA vaccine did not affect the normal functions of the colon and small intestine (fig. 2c).

Effect of the hCLCA1 DNA Vaccine on Mucus Production in Asthmatic Mice

Goblet cells are the major mucin-producing cells in the airways in asthma. The PAS stain specifically binds to mucus glycoconjugates and glycoproteins and appears as bright purple [21]. Compared with the sham-challenged group, goblet cells in the bronchial epithelium, which were stained either singly or in clusters, were obviously increased in the groups of OVA-challenged mice that received mCLCA3-p, the vector and PBS. A large amount of mucus was also found in the bronchial lumen. However,

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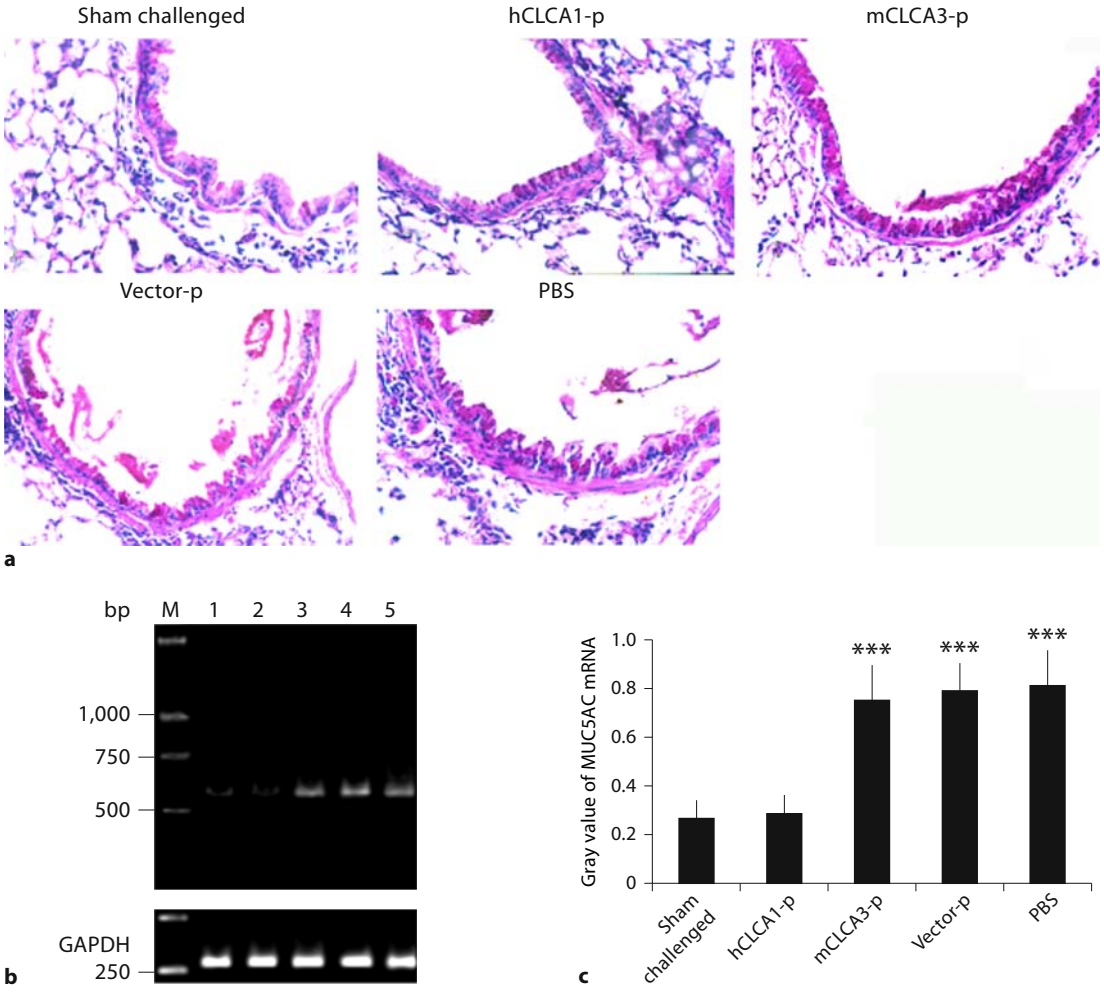


Fig. 3. Effect of the hCLCA1 DNA vaccine on mucus production in asthmatic mice. **a** PAS staining for mucus production of goblet cells in the bronchial epithelium (×200). **b** RT-PCR detection of the MUC5AC mRNA level in lung tissue. M = DNA marker; 1 = sham-challenged group; 2 = hCLCA1-p group; 3 = mCLCA3-p group; 4 = vector-p group; 5 = PBS group. GAPDH mRNA is shown as the loading control. **c** The ratio of gray value of MUC5AC mRNA. * $p < 0.01$ vs. sham-challenged group; ** $p < 0.01$ vs. hCLCA1-p group.

mucus production in the hCLCA-p group showed the same characteristics as the sham-challenged group. This observation suggests that the antibodies induced by hCLCA1-p vaccination attenuated mucin production. The reduced production of mucin could be considered as an indicator of a hypoinflammatory state (fig. 3a). MUC5AC is a gel-forming glycoprotein produced by gastric and respiratory tract epithelial cells. It has been reported that mCLCA3 or hCLCA1 can significantly induce MUC5AC expression, which is also a marker of goblet cell metaplasia in asthmatic airways. The mRNA level of MUC5AC in the challenged groups was determined by RT-PCR; the results show that the MUC5AC mRNA level increased in the mCLCA3-p, vector and PBS groups, but not in the hCLCA1-p group (fig. 3b). In addition, there was a significant decrease in the hCLCA1-p group compared with the mCLCA3-p, vector and PBS groups, and there is no significant difference between the hCLCA1-p group and the sham-challenged group (fig. 3c). These results are consistent with the results of PAS staining (fig. 3a).

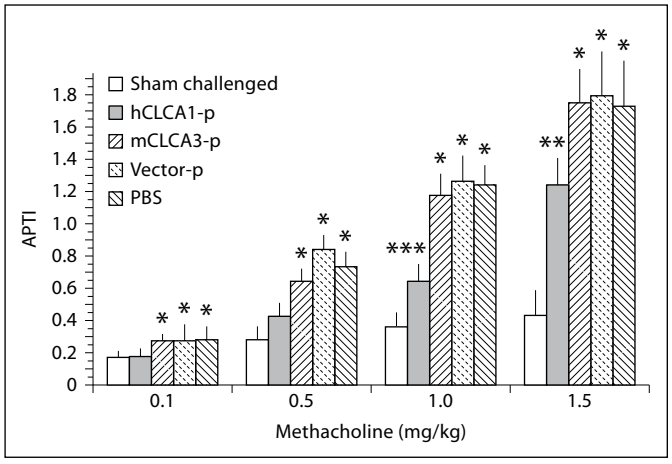


Fig. 4. Airway responsiveness of asthmatic mice to various concentrations of methacholine. * $p < 0.01$ vs. hCLCA1-p group; ** $p < 0.01$, *** $p < 0.05$ vs. sham-challenged group.

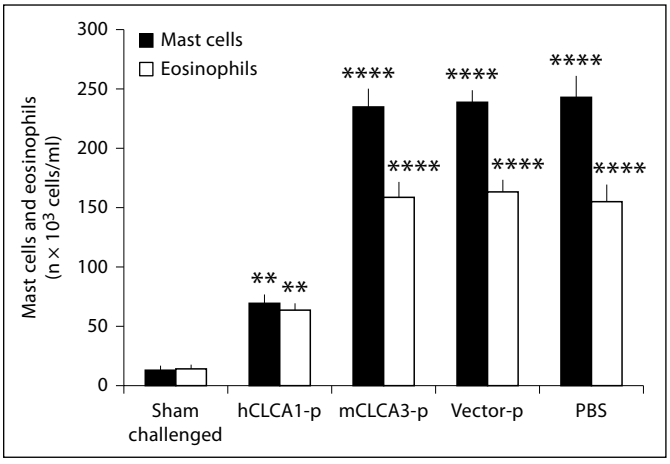


Fig. 5. The number of eosinophils and mast cells in the bronchoalveolar lavage fluid counted under a microscope after Giemsa staining. * $p < 0.01$, ** $p < 0.05$ vs. sham-challenged group; *** $p < 0.01$ vs. hCLCA1-p group).

Effect of the hCLCA1 DNA Vaccine on Airway Hyperreactivity

Airway hyperresponsiveness to nonspecific stimuli is one of the characteristic features of allergic bronchial asthma. Elevated contractility of bronchial smooth muscle is considered to be the main cause of airway hyperresponsiveness. APTI is a simple and repeatable method for testing peak inspiratory pressure and is highly correlated with respiratory resistance. The APTI-methacholine dose-relationship histogram (fig. 4) indicates that in all challenged groups APTI was significantly higher than in the sham-challenged group. However, APTI in the hCLCA1-p group was much lower than that of the other challenged groups, suggesting that a hyporeactive state existed in the mice of the hCLCA1-p group compared with the other challenged groups.

Effect of the hCLCA1 DNA Vaccine on Eosinophilic Airway Inflammation

Recruitment of eosinophils, which participate in the inflammatory process, is a hallmark of allergic asthma. There is a feedback control mechanism between eosinophils and mast cells. Mast cells play an important role in allergic asthma. The results of our study show that the number of eosinophils and mast cells in the bronchoalveolar lavage fluid was elevated in all the OVA-challenged groups. However, the number of eosinophils in the hCLCA1-p group was lower than in the other three challenged groups. The eosinophil numbers in the mCLCA3-p, vector and PBS groups were very close and were nearly threefold higher than in the hCLCA1-p group. Moreover, the number of mast cells in the four groups have the same variation tendency with the eosinophil number (fig. 5), indicating the preventive effect of hCLCA-p vaccination on asthma progression.

Discussion

The highest expression of hCLCA1 was in the small intestine, appendix and colon, whereas the majority of the hCLCA1 mRNA was in cells at the base of the crypts, with the goblet cells having the highest expression [22]. Airway goblet cells play an important role in the airways of patients with bronchial asthma, and hCLCA1 expression in the airways has been reported more recently [23]. Several studies have shown that the levels of hCLCA1 mRNA and protein are significantly increased in the airway epithelium of asthmatic patients and cleaved CLCA N- and C-termini can also be detected in bronchoalveolar lavage fluid. Overexpression of hCLCA1 and mCLCA3, two orthologs of the CLCA gene family, resulted in increased mucus production in bronchial allergic asthmatic responses [6]. CLCA3 and hCLCA1 appear to be central to the development of an airway-hypersecretory phenotype. There is significant evidence that CLCA proteins play a causal role in bronchial allergic asthmatic, suggesting that these proteins may be of interest as targets for pharmacological intervention in allergic asthma [24].

DNA vaccination is a promising strategy for treating various diseases such as infection, cancer, autoimmunity or allergy. It relies on the production of specific antibodies to pathogenic molecules along with other immunological mechanisms [25]. The mechanisms underlying DNA vaccine-induced immune responses are complicated, and DNA sequences in the plasmid are known to be important in driving an effective immune response through the induction of cytokines [26].

Asthma is an airway inflammation disease. Many types of cells play a crucial role in asthma, particular eosinophils, mast cells and T lymphocytes. These cells contributed to the release of the Th2 cell-derived cytokines IL-4, IL-9 and IL-13 [27]. These cytokines mediate their effects by binding to their receptors. For example, IL-13 mediates its effects by binding to the IL-13 receptor (IL-4R/IL-13R α1) [28]. Evidence has accumulated that the associated cytokines IL-9, IL-4 and IL-13 appear to be directly linked to CLCA overexpression in the asthmatic patient and in mouse models of asthma, with signalling via the EGFR-MAP kinase pathway [29]. The precise signals that regulate mCLCA3 gene expression in mouse models of asthma are unclear. There is some evidence that CLCA proteins increase the activity of Ca^{2+}-dependent chloride channels, and inhibiting CLCA expression inhibits mucus production. Additionally, the extracellular polypeptide structure effects of Ca^{2+} ionophores on chloride conductance associated with CLCA expression invoke changes in intracellular Ca^{2+} concentration as the basis for CLCA regulation of chloride conductance. However, it is not clear why these proteins can decrease mucus secretion. Therefore, we hypothesized that DNA vaccine could produce specific antibodies in asthmatic mice and then these antibodies may influence the function of Th2 cell cytokines via blocking of the EGFR-MAP kinase pathway. Another assumption is that DNA vaccine may influence the activity of Ca^{2+}-dependent chloride channels to relieve the symptoms of asthma.

Although the focus of most DNA vaccine studies is the induction of protective immunity against infectious pathogens, important applications of this technology have also been reported in other immune-related diseases [30]. Inspired by the fact that inhibition of a pathology-related protein (CLCA) with corresponding antibodies brought about favorable outcomes in an asthma model, we developed a DNA vaccine encoding hCLCA1, a single xenogeneic gene homologous to mCLCA3, and applied it to a mouse model of asthma, with the aim of determining whether it could induce cross-reactive antibodies against the mCLCA3 protein.

It could be advantageous to distinguish the functional segment sequences of mCLCA3 for vaccine design and for the development of a strategy to prevent and treat asthma by interfering with hCLCA1. Identification of mCLCA3 epitopes is difficult due to its relatively large molecular weight [31]. To address this issue and to determine which segments are potential epitopes, we studied the amino acid sequence of mCLCA3 and found three segments which differ markedly from their hCLCA1 counterparts. Our results suggest that these amino acids in mCLCA3 molecules would be antigenic and cross-reactive to hCLCA1. Among the three amino acid segments, the N- and C-terminal (PepN and PepC) segments could induce much stronger immunoreactions, i.e. higher antibody titers than that of the middle (PepM) segment. Our study paves the way for further discovery of specific antibodies to hCLCA1.

As we know, CLCA is a large gene family which is broadly distributed in animals and humans. There are several subtypes of each family member [21, 22]. Both hCLCA1 and mCLCA3 are constitutively expressed in the intestines and both can be induced in human and mouse airway epithelial cells under inflammatory conditions. The patch clamp technique identified niflumic acid as a nonspecific inhibitor, which could not only inhibit hCLCA1 and other chloride channels, but also activate some types of potassium channels in nerve terminals [23]. However, we are not certain whether the hCLCA1 DNA vaccine can influence other subtypes or inhibit mCLCA3, which are also located in normally functioning cells, thereby resulting in side effects. Since the mice in our study did not show serious side effects and the secretory behavior in the small intestines and colon were not affected, these results suggest that the induced antibodies may have a lower affinity for other subtypes or that compensatory mechanisms may exist in intestinal goblet cells.

In conclusion, successful vaccine design and construction made it possible for us to evaluate its efficacy in mice. Our animal experiments showed that the hCLCA1-p vaccine can reduce asthma symptoms in a mouse model, including significant inhibition of eosinophils and mast cell infiltration and effective reduction of hyperresponsiveness following methacholine challenge. Meanwhile, mucus hypersecretion was also ameliorated. The results of our study not only show that hCLCA1 is a potential therapeutic target for the prevention of human asthma, but also demonstrate the effectiveness of DNA vaccination in the treatment of diseases such as asthma or chronic obstructive pulmonary disease, suggesting that immu-

notherapeutic targeting of the hCLCA1 protein is a fascinating aspect of the prevention and treatment of asthma. However, there are some limitations to our study, e.g. hCLCA1 DNA vaccine did not totally prevent mice from asthma. The main reason is that the cause of airway hyperreactivity is not just goblet cell hyperplasia and the antibodies produced by the DNA vaccine block the function of mCLCA3 only to some extent. Further electrophysiological experiments studying serum antibodies blocking mCLCA3 may provide some evidence regarding the mechanisms of DNA vaccines.

Acknowledgments

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References

1 Ozier A, Bara I, Girodet PO, Marthan R, Berger P: Pathophysiology of asthma (in French). Rev Prat 2011;61:339–345.
2 Hauber HP, Lavigne F, Hung HL, Levitt RC, Hamid Q: Effect of Th2 type cytokines on hCLCA1 and mucus expression in cystic fibrosis airways. J Cyst Fibros 2010;9:277–279.
3 Zhou Y, Dong Q, Louahed J, Dragwa C, Savio D, Huang M, Weiss C, Tomer Y, McLane MP, Nicolaides NC: Characterization of a calcium-activated chloride channel as a shared target of Th2 cytokine pathways and its potential involvement in asthma. Am J Respir Cell Mol Biol 2001;25:486.
4 Loewen ME, Forsyth GW: Structure and function of CLCA proteins. Physiol Rev 2005;85:1061–1092.
5 Patel AC, Brett TJ, Holtzman MJ: The role of CLCA proteins in inflammatory airway disease. Annu Rev Physiol 2009;71:425–449.
6 Hoshino M, Morita S, Iwashita H, Sagiya Y, Nagi T, Nakanishi A, Ashida Y, Nishimura O, Fujisawa Y, Fujino M: Increased expression of the human Ca^{2+}-activated Cl-channel 1 (CaCC1) gene in the asthmatic airway. Am J Respir Crit Care Med 2002;165:1132–1136.
7 Ekberg-Aronsson M, Pehrsson K, Nilsson JA, Nilsson PM, Lofdahl CG: Mortality in GOLD stages of COPD and its dependence on symptoms of chronic bronchitis. Respir Res 2005;6:98.
8 Ekberg-Aronsson M, Lofdahl K, Nilsson JA, Lofdahl CG, Nilsson PM: Hospital admission rates among men and women with symptoms of chronic bronchitis and airflow limitation corresponding to the GOLD stages of chronic obstructive pulmonary disease – a population-based study. Respir Med 2008;102:109–120.
9 Loewen ME, Forsyth GW: Structure and function of CLCA proteins. Physiol Rev 2005;85:1061–1092.
10 Nakanishi A, Morita S, Iwashita H, Sagiya Y, Ashida Y, Shirafuji H, Fujisawa Y, Nishimura O, Fujino M: Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma. Proc Natl Acad Sci USA 2001;98:5175–5180.
11 Long AJ, Sypek JP, Askew R, Fish SC, Mason LE, Williams CM, Goldman SJ: Gob-5 contributes to goblet cell hyperplasia and modulates pulmonary tissue inflammation. Am J Respir Cell Mol Biol 2006;35:357–365.
12 Yasuo M, Fujimoto K, Tanabe T, Yaegashi H, Tsushima K, Takasuna K, Koike T, Yamaya M, Nikaido T: Relationship between calcium-activated chloride channel 1 and MUC5AC in goblet cell hyperplasia induced by interleukin-13 in human bronchial epithelial cells. Respiration 2006;73:347–359.
13 Nakano T, Inoue H, Fukuyama S, Matsumoto K, Matsumura M, Tsuda M, Matsumoto T, Aizawa H, Nakanishi Y: Niflumic acid suppresses interleukin-13-induced asthma phenotypes. Am J Respir Crit Care Med 2006;173:1216–1221.
14 Lai H, Rogers DF: New pharmacotherapy for airway mucus hypersecretion in asthma and COPD: targeting intracellular signaling pathways. J Aerosol Med Pulm Drug Deliv 2010;23:219–231.
15 Liu MA: DNA vaccines: an historical perspective and view to the future. Immunol Rev 2011;239:62–84.
16 Batas B, Schiraldi C, Chaudhuri JB: Inclusion body purification and protein refolding using microfiltration and size exclusion chromatography. J Biotechnol 1999;68:149–158.
17 Wang X, Yang Q, Wang P, Luo L, Chen Z, Liao B, Li G: Derp2-mutant gene vaccine inhibits airway inflammation and up-regulates Toll-like receptor 9 in an allergic asthmatic mouse model. Asian Pac J Allergy Immunol 2010;28:287–293.
18 Wu J, Xu J, Cai C, Gao X, Li L, Zhong N: Ag85B DNA vaccine suppresses airway inflammation in a murine model of asthma. Respir Res 2009;10:51.
19 HuangFu T, Lim LH, Chua KY: Efficacy evaluation of Der p 1 DNA vaccine for allergic asthma in an experimental mouse model. Vaccine 2006;24:4576–4581.
20 Seo KY, Jeon S, Choi SH, Chung SH: Niflumic acid reduces histamine-induced MUC5AC expression in human conjunctival epithelial cells. Ophthalmic Res 2011;46:181–186.
21 Pali-Scholl I, Yildirim AO, Ackermann U, Knauer T, Becker C, Garn H, Renz H, Jensen-Jarolim E, Fehrenbach H: Anti-acids lead to immunological and morphological changes in the intestine of BALB/c mice similar to human food allergy. Exp Toxicol Pathol 2008;60:337–345.
22 Gruber AD, Elble RC, Ji HL, Schreur KD, Fuller CM, Pauli BU: Genomic cloning, molecular characterization, and functional analysis of human CLCA1, the first human member of the family of Ca^{2+}-activated Cl-channel proteins. Genomics 1998;54:200–214.
23 Hauber HP, Manoukian JJ, Nguyen LHP, Sobol SE, Levitt RC, Holroyd KJ, McElvaney NG, Griffin S, Hamid Q: Increased expression of interleukin-9, interleukin-9 receptor, and the calcium-activated chloride channel hCLCA1 in the upper airways of patients with cystic fibrosis. Laryngoscope 2003;113:1037–1042.
24 Patel AC, Brett TJ, Holtzman MJ: The role of CLCA proteins in inflammatory airway disease. Annu Rev Physiol 2009;71:425–449.
25 Klinman DM, Klaschik S, Tross D, Shirota H, Steinhagen F: FDA guidance on prophylactic DNA vaccines: analysis and recommendations. Vaccine 2010;28:2801–2805.
26 Sato Y, Roman M, Tighe H, Lee D, Corr M, Nguyen MD, Silverman GJ, Lotz M, Carson DA, Raz E: Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science 1996;273:352–354.
27 Barnes PJ: Th2 cytokines and asthma: an introduction. Respir Res 2001;2:64–65.
28 Wills-Karp M: Interleukin-13 in asthma pathogenesis. Immunol Rev 2004;202:175–190.
29 Rogers DF: The airway goblet cell. Int J Biochem Cell Biol 2003;35:1–6.
30 Chua KY, Kuo IC, Huang CH: DNA vaccines for the prevention and treatment of allergy. Curr Opin Allergy Clin Immunol 2009;9:50–54.
31 Thai P, Chen Y, Dolganov G, Wu R: Differential regulation of MUC5AC/Muc5ac and hCLCA-1/mGob-5 expression in airway epithelium. Am J Respir Cell Mol Biol 2005;33:523–530.