

A Recombinant DNA Plasmid Encoding the Human Interleukin-5 Breaks Immunological Tolerance and Inhibits Airway Inflammation in a Murine Model of Asthma

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Key Words

Asthma · Eosinophils · Interleukin-5 · Airway hyperresponsiveness · Allergy

Abstract

Background: Eosinophils play a pivotal role in the generation of asthma inflammation. Interleukin (IL)-5 is the major activator of eosinophils. We hypothesize that modulating IL-5 activity could be an effective strategy for asthma therapy. In this study, we tested whether the plasmid encoding human IL-5 as a xenogeneic DNA vaccine could induce the production of autoantibodies, and be used for asthma treatment. **Methods:** A eukaryotic plasmid encoding the human IL-5 was constructed, and used as a DNA vaccine. A mouse model of asthma was established to observe its antiasthma activities. Eosinophils in tissue, blood and the bronchoalveolar lavage were stained and counted. Airway hyperresponsiveness (AHR) was determined by whole body plethysmography. Antibody characters and cytokines were detected with immunological methods. **Results:** Immunization with a plasmid encoding the human IL-5 as DNA vaccine reduced airway inflammation, reversed Th2 cytokines, and decreased AHR in mice. In addition, this immunization induced the production of polyclonal antibodies that were cross-reactive with native murine IL-5, and IgG1 and IgG2a were the major

subclasses. Adoptive transfer of the purified antibodies from the sera of mice immunized with the plasmid encoding the human IL-5 resulted in similar antiasthma effects. **Conclusions:** Our results suggest that active vaccination against IL-5 may be a rational therapeutic approach for the treatment of asthma and potentially other eosinophilic disorders.

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Introduction

Allergic asthma is a disease characterized by elevated levels of allergen-specific immunoglobulin E, chronic airway inflammation, reversible airway obstruction, and airway hyperresponsiveness (AHR) to various bronchoconstrictive stimuli [1–3]. At present, standard asthma therapies, as suggested by the management guidelines published by the National Heart, Lung, and Blood Institute [4], include oral and inhaled corticosteroids, short-acting and long-acting β -agonists, leukotriene antagonists, cromolyn, and nedocromil. Although these agents are generally successful at controlling asthma symptoms, a significant number of patients continue to have persistent symptoms, frequent exacerbations, and objective pulmonary abnormalities. In addition, the long-term use of oral and high-dose inhaled corticosteroids can cause

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significant side effects. Thus, it is essential to find alternative therapeutic approaches to allow a more effective treatment of allergic asthma.

Recent studies demonstrated that airway inflammation is the major contributing factor to asthma pathogenesis and pathobiology. The levels of airway inflammation were often correlated to the severity of the clinical symptoms and the degrees of airway obstruction and AHR [5, 6]. It is now widely recognized that eosinophils are the major cell type that plays a pivotal role in the generation of asthma inflammation. The levels of eosinophils and their inflammatory products in the lung were significantly correlated with disease severity [7]. In addition, T helper cell 2 (Th2) effector response to some common but normally innocuous environmental allergens also contributes to the generation of asthma inflammation [8–10]. Th2 cells modulate disease progression and AHR by orchestrating allergic inflammation of the airways through the release of a series of proinflammation cytokines, such as interleukin (IL)-4, IL-5, IL-10, and IL-13 [11–14]. Among these cytokines, IL-5 is thought to be the major proinflammatory pathway in asthma. IL-5 can enhance the differentiation, activation, expansion, mobilization, and in situ survival of eosinophils [15–17]. Thus, it is possible that blocking or depleting IL-5 from asthmatic patients could result in both allergic inflammation and symptom remission. Indeed, several studies have shown that passive administration of anti-IL-5 monoclonal antibodies not only inhibited the recruitment of eosinophils from the bone marrow into the targeted organs, but also attenuated allergic inflammation and clinical symptoms [18–22]. In addition, Hertz et al. [23] demonstrated that a DNA vaccine encoding murine IL-5 modified to contain a promiscuous foreign Th epitope bypassed the B cell tolerance to IL-5 and induced neutralizing polyclonal anti-IL-5 antibodies, which subsequently reduced airway inflammation and prevented the development of eosinophilia, both hallmark features of asthma in animal models and humans. These results suggest that IL-5 can be a potential target for active immunotherapy of allergic asthma.

Several recent studies have shown that immunization with xenogeneic molecules in a species could induce immune reactions against its homologous counterparts in other species by cross-reactions in tumor models [24–26]. In this study, we first compared the human IL-5 (GI 28559032) with the mouse IL-5 (GI 6754335) and found that they shared 78% nucleotide sequence identity. Thus, we hypothesized that active immunotherapy of allergic asthma with the human IL-5 as a vaccine could also be

reached through the cross-reaction between the human and mouse IL-5. In the current investigation, we extended these studies and tested the hypothesis by using a eukaryotic plasmid encoding the human IL-5 gene as a xenogeneic homologous DNA vaccine to break the immunological tolerance to IL-5 in mice. The human IL-5 DNA vaccine induced an immune response directed against the mouse self-IL-5, and reduced both pulmonary lymphocyte infiltration and eosinophilia, reduced the levels of Th2 cytokines, and inhibited the development of AHR. Our findings demonstrated that active immunogenic therapy with xenogeneic homologous DNA vaccination against IL-5 is a potential therapeutic approach for the treatment of allergic asthma and other allergic diseases.

Material and Methods

Preparation of the DNA Plasmid Vaccines

The human and murine IL-5 genes were amplified from the plasmids pORF9-hIL-5 and pORF9-mIL-5 (InvivoGen, San Diego, Calif., USA), respectively. The upstream and downstream primers for the amplification of the human IL-5 gene were 5'-TA-CAAGCTTTGAGATCACCGGTAGGAGGGCCAT-3' and 5'-TACGATATCTTAGCTGCAACAACCAAGTTTAGTCTCA-3', respectively. The upstream and downstream primers for the amplification of the murine IL-5 gene were 5'-ATTAAGCTTATCACCGGTAGGAGGGCCATC-3' and 5'-CAAGATATCTTACCATGGAGCAGCTCAGCCTCG-3', respectively. The amplified products were then subcloned into pcDNA3.1 (+) vectors (Invitrogen, Chatsworth, Calif., USA). These two recombinant plasmids (named pHIL-5 and pmIL-5, respectively) were then confirmed by sequencing and found to be identical to those in the GeneBank (GI: 28559032 for human and GI: 6754335 for murine). In addition, the empty pcDNA3.1 (+) plasmid was also used as a negative control. The recombinant and empty plasmids were amplified and purified by using two rounds of passage over Endo-Free columns (Qiagen, Chatsworth, Calif., USA). The expressions of the plasmids were confirmed in transfected COS-1 cells using RT-PCR and anti-IL-5 monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) in Western blot.

Western Blot Analysis

Western blot analysis was performed as described previously [27]. Briefly, COS-1 cells were plated in 6-well plates at 2×10^5 cells/well, and transfected with 2 μ g of plasmid (hIL-5, mIL-5 or ePlas) and 4 μ g lipofectin (Life Technologies, Grand Island, N.Y., USA) in serum-free Dulbecco's modified Eagle's medium following the manufacturer's instructions. After the addition of the lipofectin reagent-DNA complex, the cells were incubated at 37°C for 12 h. The lipofectin reagent-DNA complex-containing medium was then replaced with a normal growth medium, and the cells were further incubated at 37°C for 60 h before the conditioned medium was collected. Cell lysates were mixed with 2 $\times$ sodium dodecyl sulfate (SDS) sample buffer and electrophoresed on a 12% SDS-PAGE gel. The proteins in the gels were transferred

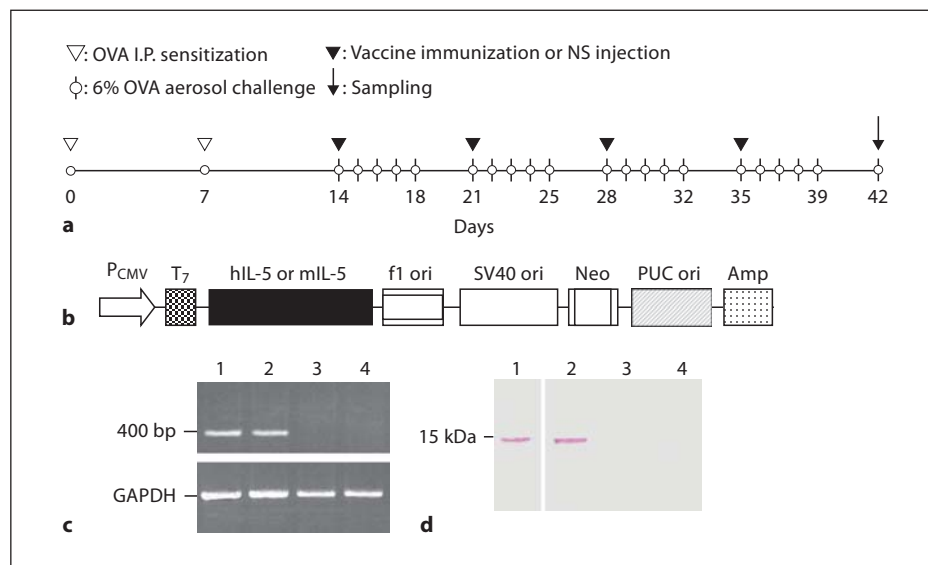


Fig. 1. Animal sensitization protocols and the construction and in vitro expression of the recombinant hIL-5 and mIL-5 plasmids. **a** Schematic representation of the setting of asthma models and vaccine treatments. **b** Human or murine cDNA clone encoding corresponding IL-5 was inserted into pcDNA3.1(+). **c** RT-PCR analysis of the expression of the recombinant plasmids. Total RNA extracted from COS-1 cells transfected with hIL-5 (**c**, lane 1), from the cells transfected with mIL-5 (**c**, lane 2), from the cells transfected with empty control pcDNA3.0 (ePlas; **c**, lane 3), and

not transfected COS-1 cells (NS; **c**, lane 4); results of RT-PCR showed that cDNA existed in the hIL-5 and mIL-5 transfected COS-1 cells (**c**, lanes 1 and 2). **d** Western blot analysis of the expression of the recombinant plasmids. Cell lysate was obtained from COS-1 cells transfected with hIL-5 (**d**, lane 1), from the COS-1 cells transfected with mIL-5 (**d**, lane 2), from the COS-1 cells transfected with ePlas (**d**, lane 3) and from the nontransfected cells (**d**, lane 4). Target proteins were expressed in the COS-1 cells transfected with hIL-5 (**d**, lane 1) and mIL-5 (**d**, lane 2).

onto a PVDF membrane. The membrane blots were blocked at 4°C in 5% nonfat dry milk, washed, and probed with anti-IL-5 antibodies (Santa Cruz Biotechnology) or sera (1:200) from the immunized mice. Blots were then washed and incubated with a biotinylated secondary antibody and stained with Vectastain ABC (Vector Laboratories, Burlingame, Calif., USA).

Animals

Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. Experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of Hainan Medical College. Specific pathogen-free male BALB/c mice between 6 and 8 weeks of age were purchased from the Experimental Animal Center of Hainan Province, People's Republic of China. The mice were housed in macrolon cages in a laminar flow cabinet and provided with ovalbumin (OVA)-free food and water ad libitum.

Mouse Asthma Model and Vaccine Immunization

To establish the mouse allergic asthma model, mice were sensitized to OVA (Sigma-Aldrich) by 2 intraperitoneal injections (7 days apart) of 100 µl alum-precipitated antigen composed of 10 µg OVA absorbed onto 2.25 mg alum (ImjectAlum; Pierce Biotechnology, Rockford, Ill., USA). Thereafter, the animals were exposed to aerosol challenge containing 6% OVA (6 mg/ml saline) for 60 min/day. The aerosol challenge was performed in a Plexi-

glas exposure chamber coupled to a Pari LC Star nebulizer (particle size 2.5–3.1 µm; PARI Respiratory Equipment, Richmond, Va., USA) driven by compressed air at a flow rate of 6 liters/min; the detailed protocols are depicted in figure 1a.

To test the therapeutic antiasthma effects of our treatments, mice were randomly divided into four groups of 10 mice each. Group 1 (xenogeneic treatment, hIL-5) mice were first sensitized with OVA at day 0 and day 7, and then challenged with 6% OVA aerosol and immunized with 100 µg of hIL-5 DNA vaccine in 100 µl normal saline (NS) by intramuscular injection in both quadriceps once weekly for 4 continuous weeks at day 14. Group 2 (homologous control, mIL-5) mice were sensitized with OVA, and then challenged with 6% OVA aerosol and immunized with 100 µg of mIL-5 as in group 1. Group 3 (empty plasmid control, ePlas) mice were sensitized with OVA, and then challenged with 6% OVA aerosol and immunized with 100 µg of empty pcDNA plasmid as in group 1. Group 4 (untreated control, NS) mice were sensitized with OVA, and then challenged with 6% OVA aerosol and injected with 100 µl NS by intramuscular injection as in group 1 (fig. 1a).

Because the OVA challenge in the current study was at a relatively high concentration, it is possible that the inflammation shown in the lung was due to the specific immunity to OVA or nonspecific irritant induced by the high-dose protein. To exclude this possibility, 10 additional mice were divided into two groups (5 mice each). One group was only sensitized with OVA and the

other group was only challenged with OVA aerosol, as mentioned before in the experimental groups. At the end of the experiments, lung tissues were collected and studied.

Lung Histology

For histological evaluation of lung tissues, lungs were removed and fixed in 10% phosphate-buffered formalin. The left lobe of each lung was embedded in paraffin, sectioned at 3–5 μm , and stained with hematoxylin and eosin. Cellular infiltrates were assessed in five randomly selected fields with a microscope at $\times 200$ magnification. Groups were analyzed in a blind fashion as done previously [28].

Bronchoalveolar Lavage

On day 42, after 6% OVA challenge, mice were sacrificed by CO_2 asphyxiation, and samples of bronchoalveolar lavage fluid (BALF) and lung tissue were collected as previously described [13]. Briefly, ten lungs per group were perfused through the pulmonary artery and lavaged through the trachea 3 times with 1 ml of PBS. Total BALF cells were determined using a hemacytometer. BALF cells were then spun onto slides by cytocentrifugation, then fixed and visualized with Hansel stain (Lide Laboratories, Florissant, Mo., USA). Differential cell counts were made on at least 400 cells using standard morphological criteria [28].

Quantification of Eosinophils

Peripheral blood samples, lung tissues and BALF were collected at the end of the experiments. Carbol's chromotrope-hematoxylin stain was used for the identification of eosinophils. Eosinophils in the peripheral blood, BALF, and lung tissues were identified based on morphological characteristics and quantified as previously described [28].

Measurement of AHR

AHR was assessed by methacholine-induced airflow obstruction from conscious mice placed in a whole body plethysmograph (model PLY 3211, Buxco Electronics, Troy, N.Y., USA). Measurements of a dimensionless parameter known as the enhanced pause (Penh) were performed as previously described [29, 30]. Briefly, mice were placed in the plethysmograph chamber and exposed to an aerosol of NS water (baseline readings) and then to cumulative concentrations of β -methacholine ranging from 3 to 50 mg/ml. The aerosol was generated by an ultrasonic nebulizer and drawn through the chamber for 2 min. The inlet was then closed, and Penh readings were taken for 3 min and averaged. The values from 10 normal mice (without sensitizing and aerosol challenging with OVA, no vaccine immunization) of the same age were used for the baseline data. Values from the above-mentioned four experimental groups were reported as the percent increase over the baseline values from the normal mice at corresponding time points.

Measurement of Th Cytokines

Th2 cytokine IL-4 and Th1 cytokine IFN- γ were detected as described previously [23, 30]. Briefly, cells from the peribronchial lymph nodes were isolated and stimulated with 1 mg/ml OVA in MLC medium for 72 h. The concentrations of IL-4 and INF- γ in the cell-free supernatants were measured by ELISA, using a mouse IL-4 ELISA kit (BMS613, Bender MedSystems, Vienna, Austria) and a mouse INF- γ ELISA kit (BMS606, Bender MedSystems),

respectively. The ELISA protocols were performed according to the instructions in the attached manuals, and the plates were measured by a Microplate reader (Bio-Tek Instruments, Winoski, Vt., USA) at 450 nm.

Determination of Autoantibodies against Self-IL-5 in the Mouse Sera

The autoantibodies against self-IL-5 in the sera were determined by ELISA as described previously [27]. Briefly, COS-1 cells transfected with hIL-5 or mIL-5 were washed with PBS and lysed by three cycles of freezing and thawing. Ninety-six-well plates were coated with 50 μl of cell lysates (1×10^5 cell equivalents) at 4°C overnight. The wells were then washed with PBS and blocked with blocking buffer at 37°C for 2 h. After washing, experimental mouse sera were serially diluted in blocking buffer and added to the wells. Plates were incubated at 37°C for 2 h, washed, and then incubated with serially diluted alkaline phosphatase-conjugated antibody against the mouse IgG subclass, IgM, or IgA. The enzymatic activities were measured with an ELISA reader (Bio-Tek).

Determination of Anti-IL-5 Antibody-Secreting B Cells

The enzyme-linked immunospot (ELISPOT) assay for the enumeration of anti-IL-5 antibody-secreting B cells (ASBCs) was performed as described previously [31]. Briefly, PVDF-bottomed 96-well filtration plates (Millipore, Bedford, Mass., USA) were coated with transfected COS-1 cell lysates. Mononuclear cells prepared from immunized mouse spleens were incubated on the plates at 37°C for 5 h. IgG bound to the membrane was revealed as spots with alkaline phosphatase-conjugated anti-mouse IgG antibodies.

Adoptive Transfer of Antibodies in vivo

Antibodies were purified from the pooled sera derived from the mice at day 7 after the fourth immunization by affinity chromatography (CM Affi-Gel blue gel kit; Bio-Rad), following the protocol described previously [25]. To assess the efficacy of antibodies in antiasthma activity in vivo, the purified antibodies (50 mg/kg) were intravenously transferred 1 day before mice were challenged with 6% OVA aerosols as done in the protocol (fig. 1a) and then treated twice per week until the last day when the mice were sacrificed. At that time, the lung histology, eosinophils, AHR and cytokines were evaluated as described above.

Statistical Analysis

The significance of differences between experimental groups was analyzed using ANOVA and/or the Student unpaired t test. Differences in means were considered significant at $p < 0.05$.

Results

Expression of the Recombinant Plasmids in vitro

The PCR-amplified DNA products encoding the human or murine IL-5 were inserted into eukaryotic plasmid pcDNA3.1(+) (fig. 1b). The resultant recombinant plasmids (hIL-5 and mIL-5) were confirmed by sequencing (data not shown). These recombinant plasmids were then used to transfect COS-1 cells and the expression of

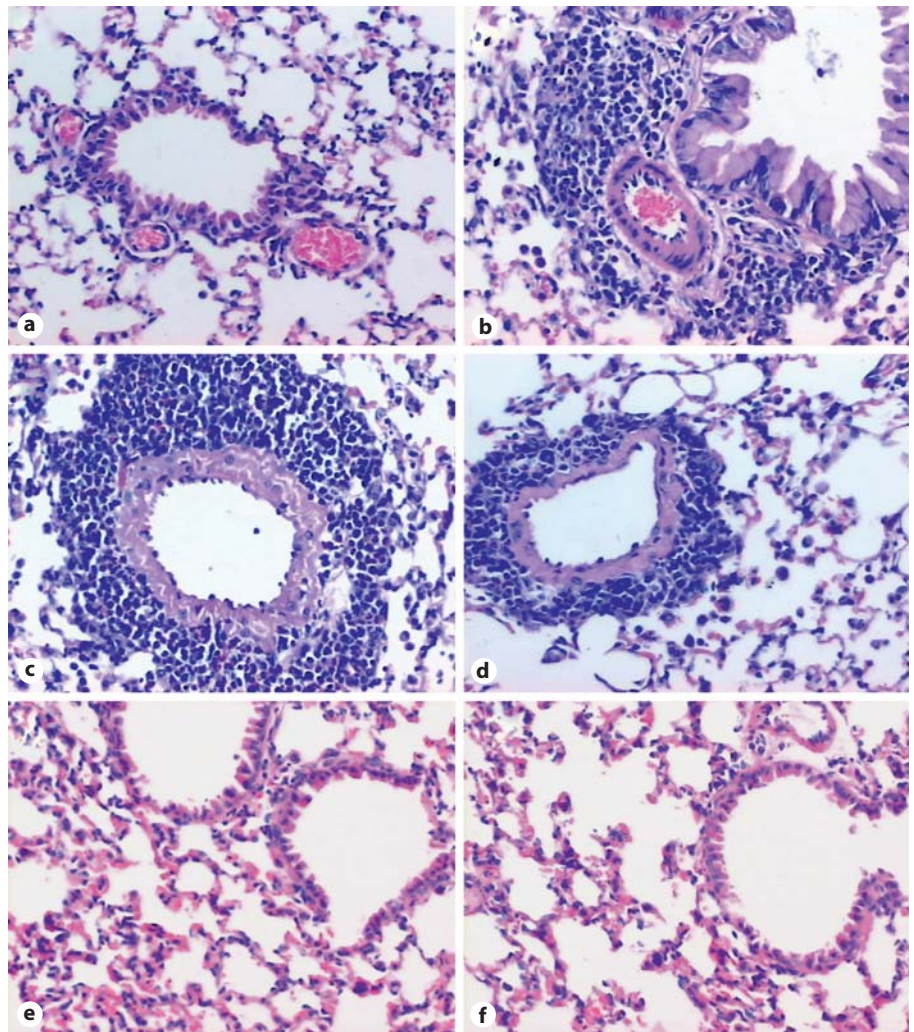


Fig. 2. Lung histology. Lung tissues were fixed in formalin and stained with hematoxylin and eosin at the day of sacrifice. Lung tissues from mice immunized with hIL-5 had only minimal inflammation (**a**). Lung tissues from mice immunized with mIL-5 (**b**), with ePlas (**c**) and with NS (**d**) revealed significant inflammation, dense peribronchiolar mononuclear cell infiltrates consisting of lymphocytes, eosinophils, and some neutrophils. In addition, lung tissues from mice only sensitized (**e**) or aerosol challenged (**f**) with OVA did not show significant inflammation. HE. $\times 200$.

the plasmids was confirmed by RT-PCR (fig. 1c) with the use of anti-human and anti-mouse IL-5 antibodies in Western blot analysis (fig. 1d).

hIL-5 DNA Immunogene Therapy Reduces the Inflammation in Lung Tissues

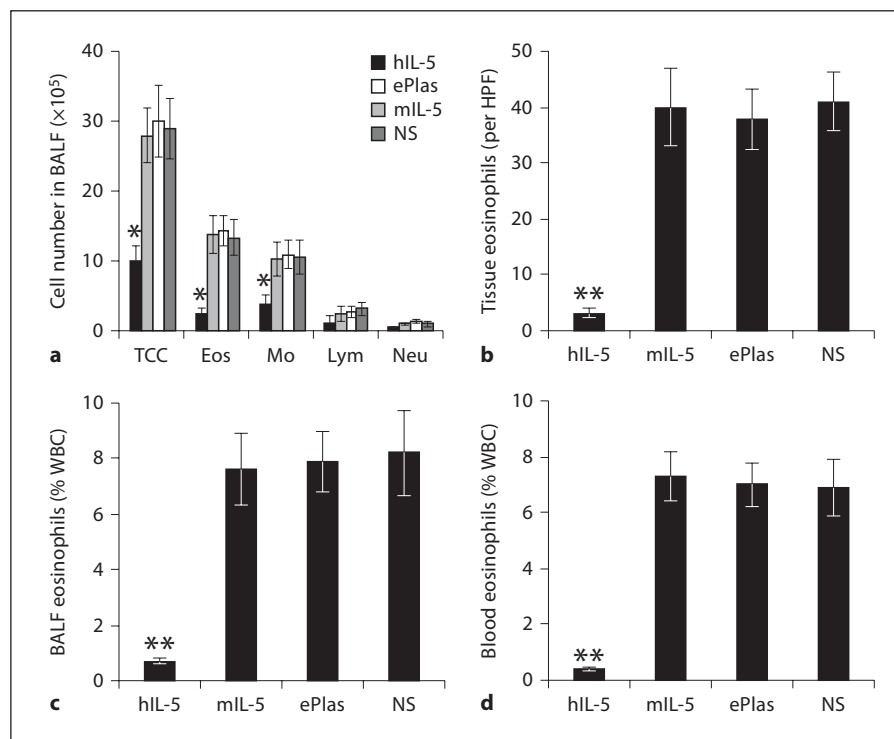
Lung sections from the mice immunized with hIL-5 showed a close to normal lung histology, with only marginal perivascular and peribronchiolar lymphocytic infiltrates (fig. 2a). In contrast, lung sections from mice immunized with mIL-5, ePlas or NS showed significant airway inflammation with peribronchiolar and perivascular infiltrates, consisting of lymphocytes, eosinophils and some neutrophils (fig. 2b–d). In addition, to exclude the possibility that the inflammation shown in the current study resulted from specific immunity to OVA or non-specific irritant induced from the high-dose OVA chal-

lenge, lung tissues from two additional groups of mice sensitized only with OVA or challenged only with OVA aerosol were studied. We found that lung tissues from both these groups of mice showed normal lung histology (fig. 2e, f) with no evidence of airway inflammation. These results demonstrated that hIL-5 DNA immunogene therapy significantly reduced airway inflammation in the mouse model of allergic asthma.

hIL-5 DNA Immunogene Therapy Reduces the Number of Eosinophils and Inflammatory Cells

BALF fluid was collected at the end of the experiment after the last aerosol challenge with OVA. We found that the total number of inflammatory cells (including eosinophils) recovered in the BALF in the mice vaccinated with hIL-5 was significantly lower than those in the three control groups (fig. 3a). In addition, the number of eo-

Fig. 3. hIL-5 DNA immunogene therapy reduces the number of inflammatory cells and eosinophils. BALF, lung tissues, and blood samples were collected as described in Material and Methods. **a** The total cell number and the relative number of eosinophils and monocytes in BALF of OVA-immunized BALB/c mice were reduced. **b** The mean number of lung peribronchial/perivascular eosinophils per 10 similar high-powered fields (HPF; $\times 1,000$ magnification). **c** The percentage of eosinophils in BALF. **d** The percentages of eosinophils in the peripheral blood. The data were expressed as mean $\pm$ SEM. $n = 5$ for each group. * $p < 0.05$; ** $p < 0.01$. TCC = Total cell count; Eos = eosinophils; Mo = monocytes; Lym = lymphocytes; Neu = neutrophils.



sinophils in BALF (fig. 3c), lung tissues (fig. 3b), and peripheral blood (fig. 3d) were also assessed by Carbol's chromotrope-hematoxylin stain in the mouse models of OVA-induced allergic asthma. Compared with mIL-5 and ePlas DNA-vaccinated or NS nonvaccinated control mice, eosinophilia from the lung tissues (fig. 3b) and blood (fig. 3d) was also significantly attenuated in the hIL-5 DNA-vaccinated mice. These results thus demonstrated that hIL-5 as a DNA vaccine significantly reduced the total inflammatory cell number and the proportion of eosinophils recovered in the BALF and blood of the mouse model of allergic asthma, confirming the results observed with lung histology.

hIL-5 DNA Immunogene Therapy Inhibits the Development of AHR

AHR was measured with a whole-body plethysmograph by challenging with increasing concentrations of methacholine at different time points. Figure 3 demonstrated the strand of AHR development. The therapeutic mice immunized with mIL-5 and ePlas, or injected with NS, developed significant AHR. However, mice immunized with hIL-5 showed dramatically reduced AHR (fig. 4), indicating that hIL-5 could significantly inhibit the development of AHR in allergic asthma mice.

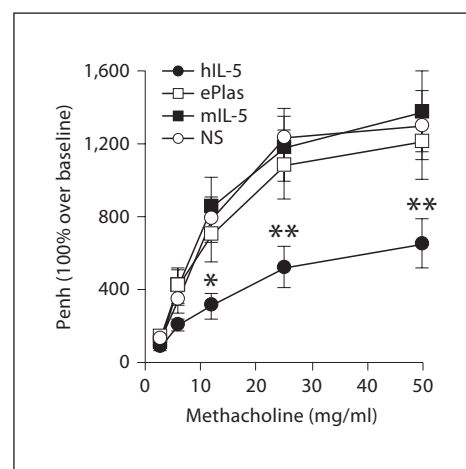


Fig. 4. Reduced AHR to cholinergic stimuli in hIL-5 DNA-vaccinated mice. Reactivity to methacholine was measured by barometric plethysmography, and the data represent the percent increase in Penh over baseline values from normal mice at corresponding concentrations of methacholine. Heightened reactivity was seen at all concentrations of methacholine in the 3 control mice, compared with that in hIL-5-vaccinated mice. * $p < 0.05$; ** $p < 0.01$.

Fig. 5. hIL-5 immunization converts an established Th2- to a Th1-like cytokine response. BALB/c mice were sensitized and aerosol challenged with OVA. Cells from the peribronchial lymph nodes were isolated and stimulated with 1 mg/ml OVA in MLC medium for 72 h. The concentrations of IL-4 (**a**) and INF- γ (**b**) in the cell-free supernatants were measured by ELISA. Data are the mean of triplicate cytokine determinations $\pm$ SEM. ** $p < 0.01$.

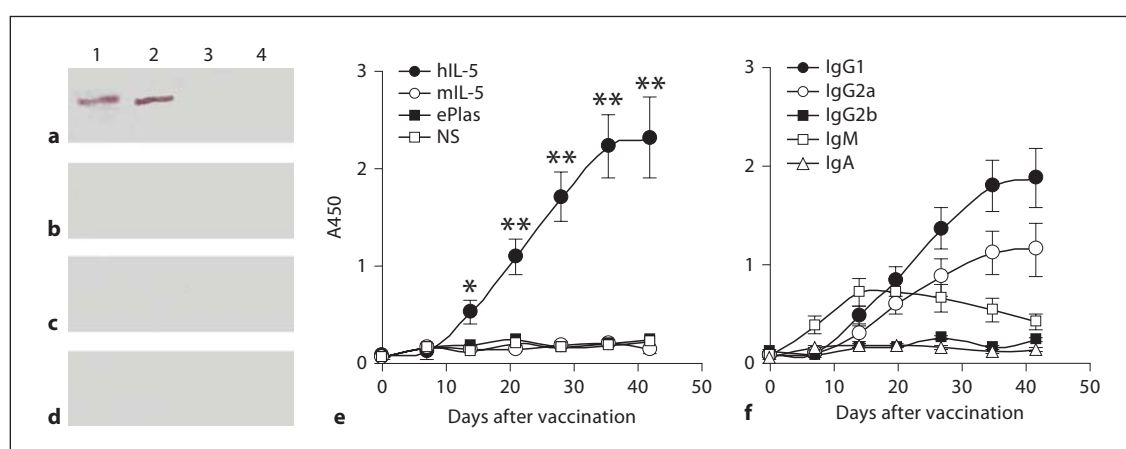
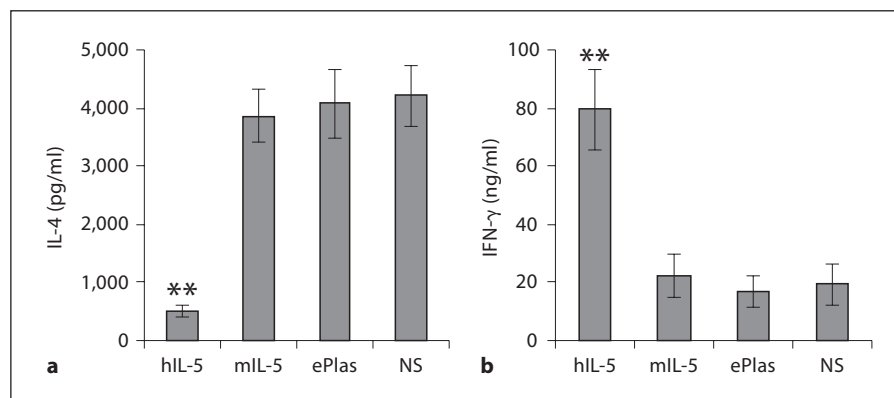


Fig. 6. hIL-5 DNA immunogene therapy bypasses B cell tolerance. **a-d** Western blotting analysis. The lysates of COS-1 cells transfected with hIL-5 (lane 1), mIL-5 (lane 2), ePlas (lane 3), or COS-1 cells alone (lane 4) were stained with sera isolated from mice immunized with hIL-5 (**a**), mIL-5 (**b**), ePlas (**c**) and NS (**d**). The positive bands were recognized with the sera isolated from mice im-

munized with hIL-5 (**a**), but negative staining comes from mice immunized with mIL-5 (**b**), ePlas (**c**) or NS (**d**). ELISA analysis also demonstrated that high levels of IgG were detected in the sera of mice immunized with hIL-5, but not in the control mice (**e**). IgG1 and IgG2b were substantially increased in the sera in the mice immunized with hIL-5 (**f**). * $p < 0.05$; ** $p < 0.01$.

hIL-5 DNA Immunogene Therapy Reverses Th2 Cytokine Production

We tested whether the reduced inflammation and AHR in allergic asthma mice immunized with hIL-5 as a vaccine were correlated with the alteration of the Th cytokine profiles (IL-4 stand for Th2 profile and INF- γ stand for Th1). At the day of sacrifice, draining lymph nodes were removed and lymph node cells were stimulated with OVA in vitro. Our data showed that cells from 3 control mice produced high levels of IL-4 (fig. 5a) and low levels of INF- γ (fig. 5b). In contrast, immunization with hIL-5 as a DNA vaccine inhibited IL-4 production and greatly enhanced INF- γ synthesis (fig. 5).

hIL-5 DNA Immunogene Therapy Bypasses B Cell Tolerance against Mouse Self-IL-5

In Western blot analysis, sera from the mice immunized with hIL-5 recognized a band with a molecular weight of about 15 kDa in COS-1 cells transfected with hIL-5 or mIL-5, but not in COS-1 cells transfected with ePlas or nontransfected COS-1 cells (fig. 6a). Sera from the other three control groups also showed negative stainings (fig. 6b-d). In ELISA, the results were similar to the Western blot results. We found that sera from the mice immunized with hIL-5 also contained the antibodies against mIL-5 (fig. 6e); the major immunoglobulin subclasses that responded to the hIL-5 were elevated signifi-

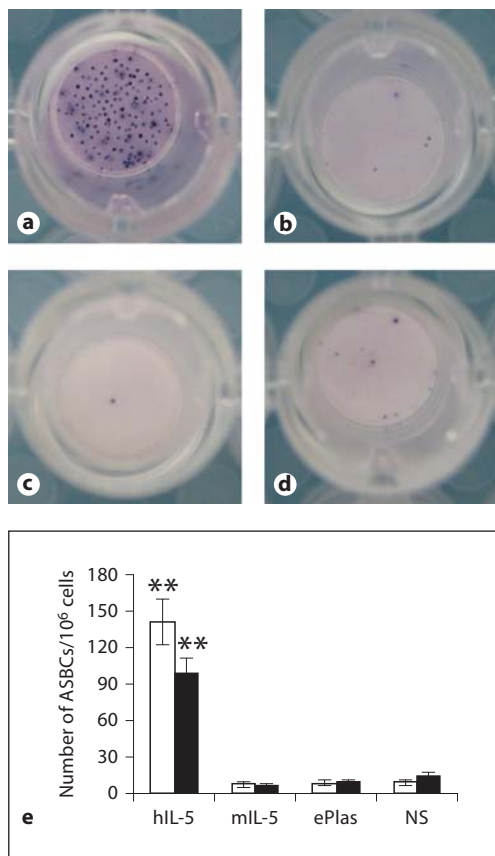


Fig. 7. hIL-5 DNA immunogene therapy induces production of anti-IL-5 ASBCs. **a–e** ELISPOT assay was used to determine the ASBCs. Mononuclear cells in mice ($n = 5$ in each group) were collected from spleens at the end of the experiment. Compared with the three control groups, the numbers of both anti-mIL-5 (□) and anti-hIL-5 (■) ASBCs were significantly increased (**e**; ** $p < 0.01$). **a–d** Four characteristic wells are shown in which anti-hIL-5 ASBCs were determined in mice immunized with hIL-5 (**a**), mIL-5 (**b**), ePlas (**c**), or NS (**d**).

cantly in IgG1 and IgG2a (fig. 6f). However, there was no antibody against either hIL-5 or mIL-5 within the sera from the control mice (data not shown). These results demonstrated that hIL-5 DNA immunogene therapy could effectively bypass B cell tolerance against mouse self-IL-5.

hIL-5 DNA Immunogene Therapy Induces Production of Anti-IL-5 ASBCs

To further characterize the mechanism of the antiasthma effects mediated by the hIL-5 DNA vaccine, the humoral immunity analysis was extended by examining the anti-IL-5 ASBCs. At the day of mouse sacrifice, we

collected the mouse spleens and studied the anti-IL-5 ASBCs by ELISPOT assay. We found that, compared with the three control groups (fig. 7b–e), the number of ASBC-secreting antibodies against hIL-5 and mIL-5 was significantly increased in the spleens of mice immunized with hIL-5 (fig. 7a, e); the level against hIL-5 was stronger than that against mIL-5, indicating that the autoantibodies against mIL-5 might be the results of cross-reaction.

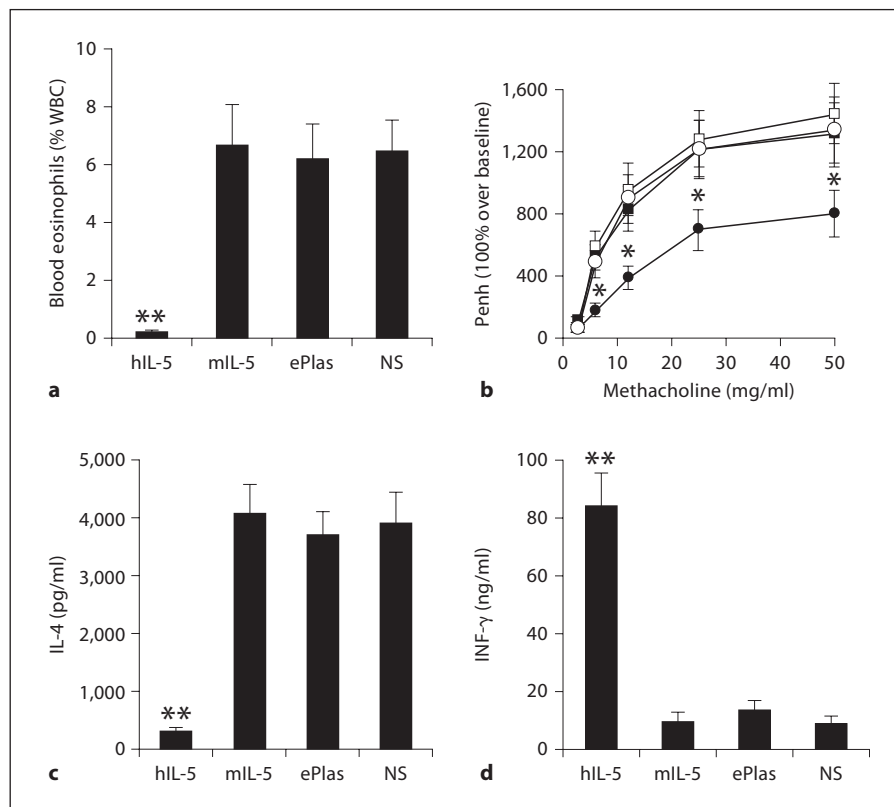
Passive Adoptive Transfer of the Purified Antibodies from the Mice Immunized with hIL-5 Induces Antiasthma Effects

To further test whether the antiasthma activities were induced by the autoantibodies against self-IL-5, passive adoptive transfer of the purified antibodies isolated from the hIL-5-immunized and the control mice was performed. Blood eosinophils, AHR and cytokines were then assayed as above. The results showed that passive transfer of the purified antibodies from mice immunized with hIL-5 also effectively reduced blood eosinophilia (fig. 8a), inhibited the development of AHR (fig. 8b), and reversed the levels of Th2 cytokines (fig. 8c, d) to the levels seen in the hIL-5 active-immunized mice. However, the adoptive transfer of the sera from the control groups did not show the antiasthma effects (fig. 8). These results indicated that the antiasthma activities of hIL-5 DNA vaccine were due to the autoantibodies against self-IL-5.

Discussion

Studies of the pathophysiology of bronchial asthma have demonstrated that airway inflammation is the key component of the disease [5, 6]. A number of inflammatory cell types, including CD4⁺ T cells and mast cells, are found in both the bronchial mucosa and the lumen of the airways, among which activated eosinophils appear to be the predominant cell type [7, 32–34]. Activated eosinophils produce a host of cytotoxic proteins such as the major basic protein, eosinophil peroxidase and eosinophil cationic protein. These proteins have been hypothesized to act in concert and contribute to the tissue edema, epithelial damage, bronchial hyperresponsiveness, mucus secretion, and airway remodeling [35]. In addition to airway eosinophilia, asthmatic individuals also display a systemic eosinophilia [7, 36], indicating that pulmonary eosinophilia is dependent on increased eosinophil production in the bone marrow prior to the release of mature cells into the bloodstream, and their subsequent recruitment to the sites of inflammation. These data demonstrated that in-

Fig. 8. Adoptive transfer of the purified antibodies induces antiasthma effects. The therapeutic antiasthma effect by adoptive transfer of immunoglobulins was tested with purified immunoglobulins (50 mg/kg) from mice (n = 5 for each group) immunized with hIL-5, mL-5, ePlas, or NS. Blood eosinophils, AHR, IL-4 and INF- γ were tested in the same way as above. Compared with the three control groups, treatment with immunoglobulins isolated from mice immunized with hIL-5 also showed reduced blood eosinophils (a), decreased AHR (b), decreased IL-4 (c), and increased INF- γ (d). These results were similar to those found in the mice actively immunized with hIL-5. The data were expressed as mean $\pm$ SEM, but AHR was expressed as the percent increase in Penh over baseline values from normal mice at corresponding concentrations of methacholine. * $p < 0.05$; ** $p < 0.01$.



hibitors of either eosinophil production, recruitment to the airway or subsequent activation could be novel anti-inflammatory approaches to future asthma therapy.

To date, there have been few specific antagonists or molecular targets directed against eosinophils. It is thus desirable to have additional therapeutic targets. Several studies have demonstrated that the function of eosinophils is principally under the control of a subset of Th2 cell-derived cytokines including IL-5, IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Although each of these cytokines has the capability of regulating the survival, adhesion, priming and activation of terminally differentiated eosinophils [37], IL-5 alone is capable of promoting the terminal differentiation of bone marrow-derived eosinophil precursors into mature eosinophils [38]. Furthermore, while IL-3 and GM-CSF can activate a broad spectrum of hematopoietic cell types, the effects of IL-5 are restricted to cells of the eosinophil/basophil lineage [34]. Thus, blocking the activity of IL-5 seemed the most specific way of targeting the eosinophil, potentially leading to the highest therapeutic benefit with the smallest number of adverse consequences. Several studies have demonstrated that the

expression of IL-5 in the lung was inversely correlated with pulmonary dysfunction in asthma patients, and the level of expression was directly correlated with the number of eosinophils detected in asthmatic airways [9, 12, 13]. Reducing IL-5 levels also reduced AHR independently of its role in eosinophilia [39], probably through the effects of IL-5 on airway smooth muscle [40, 41]. Moreover, some preclinical and clinical studies have shown that passive immunotherapy with monoclonal antibodies against IL-5 blocked peripheral eosinophilia and reduced eosinophil recruitment into the lung in murine models of allergic asthma [18–22]. However, as we know, passive immunotherapy with monoclonal antibody needs complex manipulation and a long-term usage of the antibody in vitro, representing an expensive economic burden for asthma patients, which potentially limits its further clinical use in the future. In this regard, it is highly warranted to develop a novel approach of active immunotherapy for the treatment of asthma to avoid the shortcomings of passive immunotherapy with monoclonal anti-IL-5 antibodies.

In the current study, we constructed a plasmid DNA encoding the human IL-5 as a DNA vaccine and named

it hIL-5. Our results showed that hIL-5 was capable of inducing therapeutic antiasthma efficacies in the murine model of asthma. Treatment with hIL-5 reduced airway inflammation, the production of key pathogenic Th2 cytokines and AHR in the pulmonary compartments of mice exposed to OVA-induced models of experimental asthma. Moreover, we also showed here that hIL-5 DNA vaccines could induce an immune response to produce polyclonal antibodies that are cross-reactive with native murine IL-5. Autoantibodies against murine IL-5 were identified by Western blot analysis and ELISA and IgG1 and IgG2a were the major subclasses in response to hIL-5 vaccination. Anti-IL-5 APBCs detectable by ELISPOT assay increased significantly in the spleens of mice immunized with hIL-5. In addition, a similar antiasthma activity was induced by the adoptive transfer of the purified antibodies from the sera of mice immunized with hIL-5. On the basis of these results, we conclude that the antiasthma activity of the hIL-5 immunization likely resulted from a nonspecific production of autoantibodies against the mouse self-IL-5 molecules, and these autoantibodies inhibit the function of eosinophils, which further reduced inflammation, AHR and the production of key pathogenic Th2 cytokines, all hallmark features of asthma in animal models and humans. These results demonstrated that active vaccination against IL-5 might be a rational therapeutic approach to the treatment of allergic asthma and other eosinophilic disorders.

Generally, immune responses against self-proteins such as IL-5 are presumably difficult to induce by vaccine based on either self or syngeneic IL-5 molecules due to immunological tolerance acquired during the development of the immune system. In the current study, we found that the human and murine IL-5 genes were highly similar at the nucleotide level. Our study demonstrated that the human IL-5 was capable of inducing a B cell response that elicits cross-reactive immunity with native IL-

5 in mice, suggesting that murine IL-5, if used as an antigen, might also possibly induce a B cell response against human IL-5 and prevent and/or cure allergic asthma in human patients. In contrast to the systemic humanized anti-IL-5 monoclonal antibody administration, active immunotherapy with xenogeneic homologous IL-5 as a vaccine has several advantages as discussed above. Thus, it would be beneficial to further explore the possibility and potential mechanisms using IL-5 from mice or other species as antigens to induce antibodies against human self-IL-5 and to treat human allergic asthma. In addition, the approach developed in our current study might also allow for longitudinal immunotherapy and potential resolution from other eosinophilic disorders.

In summary, the present study showed that a recombinant plasmid encoding human IL-5 used as a murine homologous DNA vaccine effectively broke the immune tolerance against self-IL-5 in the murine allergic asthma model. Autoantibodies against self-IL-5 induced the reduction of pulmonary inflammation, shifting the cytokine production from Th2 to Th1 and inhibiting the development of AHR, which were all hallmarks of allergic asthma. Thus, our findings demonstrated that active immunogene therapy with xenogeneic homologous DNA vaccination against IL-5 could be a potential therapeutic approach to the treatment of allergic asthma and other allergic diseases.

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