



Intranasal administration of CpG oligodeoxynucleotides reduces lower airway inflammation in a murine model of combined allergic rhinitis and asthma syndrome

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ABSTRACT

Given the relationship between allergic rhinitis (AR) and asthma, it can be hypothesized that reducing upper airway inflammation by targeting oligodeoxynucleotides with CpG motifs (CpG-ODN) specifically to the upper airway via intranasal administration in a small volume (10 μ L) might improve lower airway (asthma) outcomes. The goal of this study was to investigate the therapeutic efficacy of 10 μ L of intranasal versus intradermal administration of CpG-ODN in suppressing lower airway inflammation and methacholine-induced airway hyperreactivity (AHR) in mice subjected to ovalbumin (OVA)-induced combined allergic rhinitis and asthma syndrome (CARAS). OVA-sensitized BALB/c mice were subjected to upper-airway intranasal OVA exposure three times per week for 3 weeks. Then, CpG-ODN was administered to a subset of these mice 1 h after intranasal OVA exposure, followed by five days of OVA aerosol challenges, thereby targeting OVA to the lower airways. Immunologic variables and nasal symptoms were evaluated. The results showed that the CARAS mice exhibited significant increases in bronchoalveolar lavage fluid (BALF) and splenocytes Th2-associated cytokine production, OVA-specific serum IgE, and AHR, as well as nose and lung pathologies. Intranasal administration of CpG-ODN significantly reduced Th2-associated cytokine production, the percentage of eosinophils in the BALF, the IL-4 and IL-5 concentrations in the supernatants of cultured OVA-challenged splenic lymphocytes, the serum OVA-specific IgE levels, the peribronchial inflammation score in the lungs, and the severity of nose pathology and nasal symptoms. However, intradermal administration of CpG-ODN did not significantly reduce the aforementioned parameters. In conclusion, intranasal treatment with CpG-ODN attenuated AR and significantly alleviated lower airway inflammation and AHR in the CARAS model. CpG-ODN therapy was more effective when administered intranasally than when administered intradermally. The current study supports the development of CpG-ODN nasal spray as a novel therapeutic agent for CARAS.

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1. Introduction

Although allergic rhinitis (AR) and bronchial asthma are two features of allergic diseases of the respiratory tract, accumulating data that have emerged in recent decades support an association between upper and lower airway inflammation, often referred to as 'united airways', 'combined allergic rhinitis and asthma syndrome (CARAS)' or 'one airway-one disease' [1–3]. AR and asthma are highly prevalent and often comorbid diseases [4–6]. Estimates vary based on the rigor with which AR and asthma are diagnosed; however, up to 80% of asthmatics are affected by AR, and up to 40% of patients with AR have

concomitant asthma [6]. Prior studies have shown that AR is one of several risk factors for the development of asthma in both adults and children [7,8]. One in three AR patients may progress to develop asthma within 10 years [9]. AR can complicate asthma management and lead to poor asthma control and outcomes [6]. Patient studies suggest that appropriately treating upper-airway inflammation prevents progression to asthma [10], reduces lower-airway symptomatology, and improves asthma control [11,12]. Together, these data show that AR can precede and is a risk factor for asthma. Clinical and basic science evidence indicates that these two diseases share epidemiological, anatomical, physiological, immunopathological, and therapeutic bases [3,6,13–15].

In most cases, therapies for the treatment of bronchial asthma follow the current guidelines for asthma treatment [16,17], these therapies are directed toward improving symptoms by reducing inflammation and

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reversing or inhibiting bronchospasm. Despite the existence of a variety of pharmaceutical options, including glucocorticoids and leukotriene pathway antagonists, a cure for bronchial asthma remains elusive, as there is no effective treatment that controls the underlying immune T helper (Th)1/Th2 imbalance that perpetuates allergic disorders [18]. Because AR and asthma are inflammatory disorders that exhibit a Th2-dominant immune imbalance caused by the over-production of Th2-type cytokines and a decrease in Th1 cytokine production, designing a therapeutic strategy to shift the immune response toward the Th1 type has been of special interest to researchers in recent years [19–22]. Data obtained from murine and human systems have demonstrated that oligodeoxynucleotides (ODN) with CpG motifs (CpG-ODN) engage Toll-like receptor 9 (TLR-9) on dendritic cells (DCs), B-cells and other cell types, induce T helper (Th)1-type responses [23], and concomitantly inhibit preexisting Th2 responses. It has been reported that the administration of CpG-ODN alone and the co-administration of CpG-ODN with allergens attenuate AR [24]. Considering that AR and asthma are mediated by similar allergic inflammatory mechanisms, AR might precede asthma and act as an initiator of CARAS. Thus, we speculate that treating AR with CpG-ODN might improve lower-airway inflammation in CARAS. To explore this hypothesis, we used a previously described murine ovalbumin (OVA)-induced model of CARAS [3] and investigated the effect of intranasal versus intradermal administration of CpG-ODN on CARAS BALB/c mice during antigen challenge. The results indicated that the treatment of AR with intranasal CpG-ODN alleviated lower-airway inflammation and AHR in the CARAS model. To the best of our knowledge, previous studies investigating the effects of CpG-ODN on allergic disorders have been limited to otolaryngological or pulmonary diseases. Studies concentrating on CARAS, which is a combined disease of the nose and the lower airways, are very rare.

2. Materials and methods

2.1. Mice

Female BALB/c mice (6 to 8-week-old) were obtained from Sun Yat-sen University animal center and housed under specific pathogen-free conditions with the approval of the local animal ethics and welfare committee. The animals were maintained in a 12-hour light–dark cycle and were provided with food and water at libitum. To induce AR, the BALB/c mice were sensitized on days 0 and 7 via intraperitoneal injection of OVA (10 µg OVA, grade V, Sigma, USA), which was emulsified in 2 mg of AL(OH)₃ (OVA/alum). Two weeks later, the mice were challenged while awake by instilling a 10-µL droplet of either OVA (1 mg/mL; AR group) or phosphate-buffered saline (PBS) into each nostril using a

micropipette. The mice were challenged for 3 successive days on 3 consecutive weeks according to a previously reported protocol with slight modifications [3]. Immediately after the final nasal challenge, the numbers of nose-rubbing and sneezing movements were recorded using a camcorder (SONY, Japan) for 30 min. Twenty-four hours after the final intranasal application of either OVA or PBS, the awake mice were challenged with either 5 ml of 2% OVA (CARAS group) or 5 ml of PBS using an aerosol exposure technique for 30 min on 5 consecutive days. For the CpG-ODN experiment, as indicated in Fig. 1, 1 h after the nasal challenge, the mice were administered 10 µg of CpG-ODN either intranasally (1 mg/mL) (CpG-ODN i.n. group) or intradermally (0.1 mg/mL) (CpG-ODN i.d. group), a dose that is known to be optimal for the stimulation of murine cells [25]. Synthetic CpG-ODN containing two CpG motifs (5'-TCCATGACGTTCTGACGTT-3') (endotoxin-free and higher than 99% purity; Sangon Biotechnology, Shanghai, China) were used as reported previously [26]. The optimal dosages of CpG-ODN were obtained from a pilot study using different CpG-ODN dosages (2.5, 5.0, 10.0 and 20.0 µg). The results of the pilot study showed that the optimal CpG-ODN dose was 10.0 µg. The control mice were treated in the same manner as the experimental mice, except that the administration of OVA and CpG-ODN was omitted during both the sensitization and challenge phases. The protocol used in this study is schematically depicted in Fig. 1.

2.2. Assessment of airway reactivity

Airway resistance was measured using a methacholine-induced airflow obstruction method according to a previously our described protocol, which was slightly modified [27]. Twenty-four hours after the final aerosol challenge, the mice were placed in a whole-body plethysmograph (Buxco Electronics, USA). The mice were first placed in the chamber for acclimatization. Then, the mice were treated with nebulized normal saline, followed by the administration of increasing concentrations (6.25, 12.5, 25, and 50 mg/mL) of nebulized methacholine (Sigma-Aldrich). The reading interval was set to 5 min following the nebulization of each substance at each concentration. The enhanced pause (Penh) values for each methacholine dose were determined using the following equation: $\text{Penh} = \text{pause} \times (\text{peak inspiratory box flow} / \text{peak expiratory box flow})$ [27].

2.3. Cytokine quantification and cell collection in bronchoalveolar lavage fluid (BALF)

As described in our previous study [27,28], after euthanization, each mouse was placed in a supine position with the head tilted back. The

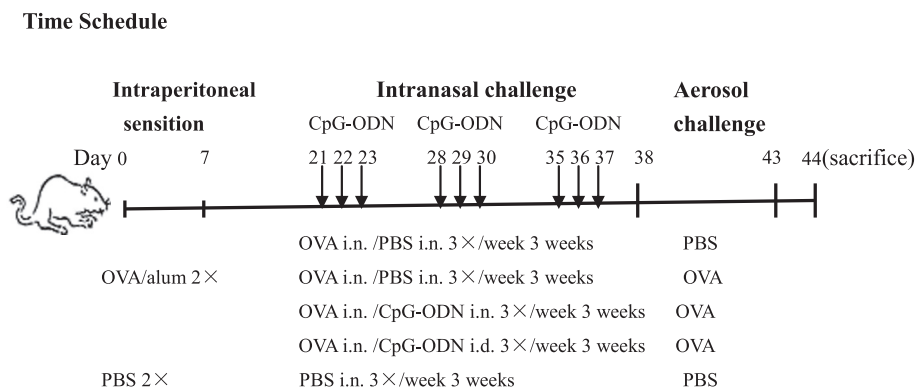


Fig. 1. Experimental protocol. Mice were developed as AR mice or as negative controls by sensitization with OVA/alum or phosphate-buffered saline (PBS) on days 0 and 7, respectively. Sensitization was followed by a nasal challenge with OVA or PBS thrice weekly for 3 weeks, with or without 10 µg CpG-ODN intranasal (i.n.) or intradermal (i.d.) applications 1 h after nasal challenge. On day 38, the mice were divided into the following groups: mice that received an OVA aerosol challenge (i.e., the CARAS model), mice that received PBS aerosol (i.e., the AR model) and control mice, which were sensitized with PBS only and then challenged with PBS aerosol. Twenty-four hours after the final aerosol challenge on day 44, the mice were sacrificed and sampled.

right major bronchus was tied with a string, and a 24-gauge needle was inserted into the bronchus to collect the BALF. The BALF was obtained via the infusion and collection of 0.5 ml of saline. The infusion and collection steps were repeated 3 times. The BALF was centrifuged at 2 000 rpm for 10 min at 4 °C, and the supernatant was stored at –80 °C for further cytokine quantification via ELISA. The differential cell counts in the BALF were performed as described in our previous study [27]. In brief, the pellet was resuspended in 0.5 ml of saline, and the differential cell counts were determined by counting at least 400 cells under a light microscope. The total cell number and the number of eosinophils were determined by counting the cell numbers in randomly selected high-power fields (40× magnification) and by dividing the number of eosinophils by the total number of cells per high-power field. All counts were performed by the same blinded observer in a randomized order. The concentrations of IL-4, IL-5, IL-13 and IFN- γ in the BALF were measured using ELISA kits (Elabsciences, China) according to the manufacturer's instructions.

2.4. *In vitro* cytokine assays from splenic lymphoid cells restimulated with ovalbumin

We evaluated the antigen-specific recall response by splenocytes cultured in the presence of antigen as described in previous studies [24,29–33]. Because there were no detectable levels of the assayed cytokines in the supernatants of splenocytes cultured in the absence of antigen [24,29,30], in our study, lymphoid cells (4×10^5 cells per 200 μ L) from the all groups of spleen were cultured with OVA (10 μ g/mL) for 72 h at 37 °C in RPMI-1640 medium (Sigma, USA) supplemented with 10% fetal calf serum (Hangzhou Sijiqing, China), 100 units/ml penicillin, and 100 μ g/ml streptomycin (North China Pharmaceutical Corporation, China). The cells were incubated at 37 °C in an atmosphere of 5% CO₂ in air for 4 days as previously described [24]. After this incubation, the cell-free culture supernatants were harvested. To measure the concentrations of IL-4, IL-5, and IFN- γ in the culture supernatants, we used commercially available ELISA kits (Elabsciences, China) in accordance with the manufacturer's instructions.

2.5. Measurement of total and OVA-specific IgE

A total of 0.7 ml of blood was collected from each mouse via cardiac exsanguination. The total and OVA-specific IgE levels were determined using ELISA kits (Elabsciences, China) according to the manufacturer's instructions.

2.6. Nose and lower airway pathology

Nasal tissue was isolated previously published methods with slight modification of [24]. Briefly, when the mice were sacrificed, the scalps were removed. After fixing in 10% buffered formalin, the heads were sectioned. Five-micrometer-thick cryosections of nasal tissue were stained with hematoxylin and eosin (H&E). The number of eosinophils was evaluated microscopically on both sides of the nasal septum. To assess lower-airway inflammation, the lungs were dissected from the chest cavity. The left lung was immediately fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with H&E or periodic acid-Schiff (PAS) to assess inflammatory changes and goblet cell metaplasia, respectively. The severity of peribronchial inflammation was scored by a blinded observer according to the following previously described criteria [34]: 0, normal; 1, few cells; 2, a ring of inflammatory cells 1 cell layer deep; 3, a ring of inflammatory cells 2–4 layers deep; 4, a ring of inflammatory cells >4 layers deep.

2.7. Data analysis

For the statistical evaluations, one-way ANOVA was used to calculate the overall *P*-value, followed by post hoc analysis using Bonferroni's test

for multiple comparisons of means. The statistical software package STATA 10.0 (Stata Corp LP) was used for the analyses. A *P*-value of <0.05 was considered to indicate a significant difference between the groups. All results are presented as the means $\pm$ SD.

3. Results

3.1. Intranasal CpG-ODN intranasal administration relieves nasal symptoms

To examine the effects of CpG-ODN on nasal symptomatology, we counted the number of nose-rubbing and sneezing movements for 30 min immediately after the final nasal challenge. After exposure to OVA, the sensitized mice developed increased nose scratching and sneezing. Compared with the control mice, the animals with AR and CARAS exhibited a significant increase in nose rubbing and sneezing (both $P < 0.01$, Fig. 2). The number of nose-rubbing and sneezing movements was significantly lower in mice with CARAS that received intranasal CpG-ODN therapy than in mice that received no therapy or intradermal CpG-ODN therapy (both $P < 0.01$, Fig. 2). However, no significant difference was observed between mice with CARAS that received intradermal CpG-ODN therapy and mice with CARAS that received no therapy.

3.2. Intranasal CpG-ODN therapy inhibits Th2-type cytokine production from BALF and splenocytes

We analyzed the levels of Th1/Th2-associated cytokines in the BALF of the experimental animals to elucidate the contribution of CpG-ODN treatment to Th1/Th2 cytokine homeostasis in this CARAS mouse model. The level of IFN- γ was elevated in all three groups (the CARAS group, the CpG-ODN i.n. group and the CpG-ODN i.d. group) compared with the IFN- γ levels in the control group ($P < 0.01$, Fig. 3A); however, the IFN- γ levels were not significantly between the three groups with CARAS. The levels of IL-4, IL-5 and IL-13 were higher in the mice with CARAS than in the control and AR animals (both $P < 0.01$, Fig. 3A). Intranasal CpG-ODN treatment significantly reduced the levels of IL-4, IL-5 and IL-13 compared to no treatment among the mice with CARAS ($P < 0.05$, Fig. 3A). A trend toward lower levels of IL-4, IL-5, and IL-13 was observed in the BALF of the animals that received intradermal CpG-ODN therapy. For comparison, the CARAS mice exhibited a significant shift in the IFN- γ /IL-4 ratio compared with the AR mice ($P < 0.01$, Fig. 3B). However, this change was significantly reversed by intranasal CpG-ODN treatment ($P < 0.01$, Fig. 3B), but intradermal CpG-ODN therapy did not result in a statistically significant difference in the IFN- γ /IL-4 ratio compared to no treatment of the mice with CARAS ($P = 1.00$, Fig. 3B).

To investigate the effects of CpG-ODN on splenocyte cytokine production, we measured the production of IL-4, IL-5 and IFN- γ by splenocytes that were stimulated with OVA *in vitro*. The mice with AR or CARAS exhibited significantly higher levels of IL-4 and IL-5 compared with the control mice (both $P < 0.01$, Fig. 3C and D). Splenocyte lymphocytes from BALB/c mice that had received intranasal CpG-ODN therapy released significantly lower levels of IL-4 and IL-5 than those from BALB/c mice that received no therapy (both $P < 0.01$). The level of IL-5 in the mice that received intradermal CpG-ODN therapy was significantly higher than that in the mice that received intranasal CpG-ODN therapy ($P < 0.01$), but there were no differences in the level of IL-4 between the groups that received intranasal or intradermal CpG-ODN therapy ($P = 0.24$, Fig. 3C and D). The levels of IFN- γ produced by splenocytes from BALB/c mice receiving either intranasal or intradermal CpG-ODN therapy were undetectable. These findings clearly indicate that a robust Th2 response was induced in the mice with CARAS and that intranasal CpG-ODN treatment markedly suppressed the dominant production of Th2-associated cytokines from the splenocytes and BALF and modulated Th1/Th2 homeostasis in the mouse CARAS model.

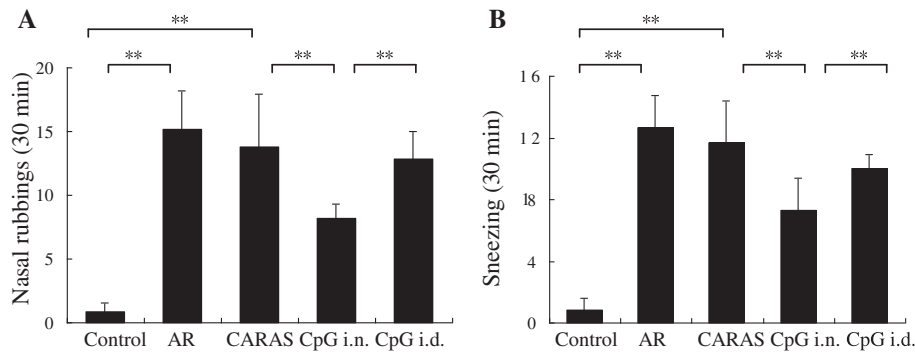


Fig. 2. Intranasal administration of CpG-ODN improved nasal symptoms. Mice received intranasal CpG-ODN (i.n.) or intradermal injections of CpG-ODN (i.d.). After the last nasal challenge, the number of nasal rubbing (A) and sneezing (B) movements was counted. The data shown represent means $\pm$ SD ($n = 6$ animals per group). ** $P < 0.01$ versus the counterpart group.

3.3. Intranasal CpG-ODN therapy reduces the serum level of OVA-specific IgE

To investigate the effect of CpG-ODN on immunoglobulin production during OVA challenge, we measured the total levels of IgE and OVA-specific IgE in serum. As indicated in Table 1, BALB/c mice that received intranasal CpG-ODN therapy exhibited significantly lower levels of OVA-specific IgE than the control mice or the untreated CARAS mice (all $P < 0.01$). However, the level of OVA-specific IgE in the BALB/c mice that received the intradermal CpG-ODN therapy was not significantly lower than that in the control or untreated CARAS mice (all $P > 0.05$). Alternatively, there were no significant differences in the total IgE level between any of the groups (except for the control group) based on Bonferroni's test for multiple comparisons of means.

3.4. Intranasal CpG-ODN therapy inhibits nasal mucosa and lower-airway cellular inflammation

To determine whether the protective effects of CpG-ODN that were observed previously in the AR model [24] would further inhibit lower-airway cellular inflammation after repeated exposure to aerosolized OVA, we evaluated nasal eosinophilia and lower-airway inflammation. We first assessed the distribution and severity of allergen-induced intranasal submucosal eosinophilic inflammation and observed that intranasal OVA challenge induced submucosal eosinophilia in the sensitized mice (Fig. 4A). The mice with AR and CARAS developed significant submucosal eosinophilia compared with the control mice (both $P < 0.01$, Fig. 4D). Intranasal CpG-ODN therapy resulted in fewer eosinophils than no treatment or intradermal CpG-ODN therapy in the CARAS mice (both $P < 0.01$, Fig. 4D).

We next examined the severity of lower-airway inflammation using the peribronchial inflammation score [34] and found that animals with CARAS exhibited robust airway inflammation, which was characterized by the influx of inflammatory cells, including eosinophils and lymphocytes (Fig. 4B). As shown in Fig. 4C, the CARAS mice showed greater goblet cell hyperplasia than the AR mice. However, CpG-ODN i.n. treatment ameliorated airway inflammatory cell infiltration and goblet cell metaplasia compared with CpG-ODN i.d. treatment (Fig. 4B and C). The inflammation score of the mice with CARAS was significantly higher than that of the mice with AR and of the control animals (all $P < 0.01$) and was significantly lower in the mice that had been intranasally treated with CpG-ODN ($P < 0.05$, Fig. 4E). However, the mice in the CARAS and intradermal CpG-ODN therapy groups were not significantly different ($p = 1.00$, Fig. 4E). Total cell number, the absolute number of eosinophils, and the ratio of eosinophils to total cells (EOS%) were higher in the BALF of the CARAS mice than in that of the controls and AR mice (all $P < 0.01$, Fig. 4F–H). Intranasal CpG-ODN therapy markedly inhibited these values compared to no treatment ($P < 0.01$), but not

intradermal CpG-ODN treatment ($P > 0.05$), except for, surprisingly, the absolute numbers of eosinophils in the CARAS mice ($P < 0.01$) (Fig. 4F–H). These data demonstrated that intranasal CpG-ODN therapy reduced not only local nasal inflammation but also lower-airway inflammation.

3.5. Intranasal CpG-ODN therapy attenuates airway hyper-reactivity to methacholine

Additionally, we determined the effect of the CpG-ODN therapy on AHR in the CARAS model by assessing Penh, which has been used to evaluate changes in pulmonary function and as a method to evaluate airway reactivity. The CARAS mice exhibited significantly greater responses and sensitivity to inhaled methacholine than the control mice and the AR mice (all $P < 0.01$, Fig. 4I). Treatment with intranasal CpG-ODN significantly decreased AHR, but no changes were observed in the Penh values of the intradermal CpG-ODN treated group (CpG i.n. vs. CARAS, $P < 0.01$; CpG i.d. vs. CARAS, $P = 0.10$, Fig. 4I). These data revealed that local intranasal CpG-ODN therapy decreased methacholine-induced AHR.

4. Discussion

The results obtained here suggest that OVA-sensitized mice, which were subjected to three weeks of continuous intranasal OVA exposure followed by five days of aerosolized OVA challenge, exhibited not only local upper-airway nasal eosinophilic inflammation but also Th2-mediated lower-airway lung inflammation, as demonstrated by large increases in the IL-4, IL-5 and IL-13 levels, eosinophilia in BALF, and the OVA-specific IgE levels in serum (Figs. 3–4, Table 1). Compared with intradermal administration, intranasal administration of CpG-ODN significantly reversed nose and lung pathologies, decreased the levels of OVA-specific IgE, ameliorated the lower-airway eosinophilic inflammation and goblet cell metaplasia, led to a reversal of Th2-type responses in splenocytes and BALF, decreased AHR in response to inhaled methacholine, and alleviated the severity of nasal symptoms in the murine model of CARAS. The results obtained lend support to the unified airway theory i.e., the hypothesis that upper-airway treatment can affect lower-airway inflammation. This finding also suggests that the local intranasal administration of CpG-ODN during allergen exposure is more effective than the systemic intradermal administration of CpG-ODN in controlling AR and asthma, particularly in the CARAS model. However, it should be emphasized that the proportion of Th1/Th2 cytokines remained shifted toward Th2 cytokines following intranasal CpG-ODN treatment compared with the control treatment (Fig. 4). This result suggests that the suppression of allergic responses was only modest. Taken together, these findings indicate that intranasal administration of CpG-ODN alone has the potential to at least partly

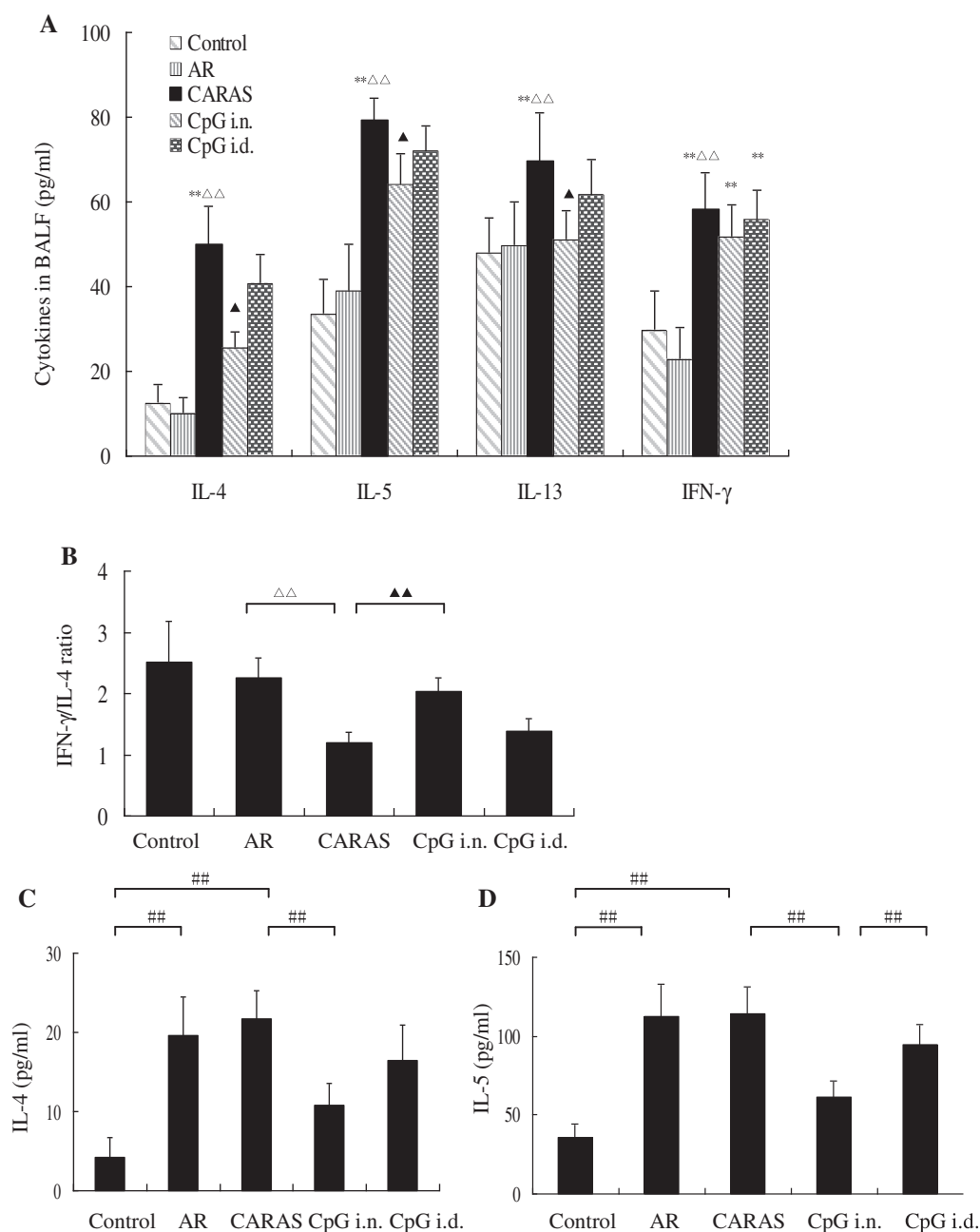


Fig. 3. Intranasal CpG-ODN therapy alters Th2-type responses from BALF and splenocytes. According to the protocol (Fig. 1), CARAS mice underwent intranasal (i.n.) or intradermal (i.d.) treatment with CpG-ODN. (A) ELISA analysis of Th1/Th2-associated cytokines levels in BALF. (B) IFN-γ/IL-4 ratio in the BALF was evaluated using ELISA assay. IL-4 (C) and IL-5 (D) production in culture supernatants of splenocytes were determined using an ELISA kit. The data shown represent means $\pm$ SD ($n = 6$ animals per group). ** $P < 0.01$ compared with the control group. $\Delta\Delta P < 0.01$ compared with the AR group. $\blacktriangle P < 0.05$ compared with the CARAS group and $\blacktriangle\blacktriangle P < 0.01$ compared with the CARAS group. $\#\# P < 0.01$ versus the counterpart group.

Table 1
Comparison of serum total IgE and OVA-specific IgE ($\bar{x} \pm s$).

Group	n	Total IgE (ng/ml)	OVA-specific IgE (ng/ml)
Control animals	6	1583 $\pm$ 381.35	10.13 $\pm$ 2.82
AR mice	6	2175 $\pm$ 285.15	104.83 $\pm$ 18.76**
CARAS mice	6	2165 $\pm$ 291.39	114.08 $\pm$ 18.86**
CpG-ODN intranasal therapy	6	2140 $\pm$ 352.48	48.38 $\pm$ 11.08** $\blacktriangle\blacktriangle$
CpG-ODN intradermal therapy	6	2072 $\pm$ 349.71	88.73 $\pm$ 17.14
F value		3.39	49.73
P value		0.02	<0.01

Values are means $\pm$ SD. ** $P < 0.01$ compared with the control group. $\blacktriangle\blacktriangle P < 0.01$ compared with the CARAS group.

inhibit lower-airway inflammation and that this therapy might serve as a novel therapeutic strategy for the treatment of CARAS.

Our observations confirm similar findings by others using a variety of allergens, animal strains, and timings of CpG-ODN administration. For example, Hussain et al. [30] found that the AR mice that received CpG-ODN exhibited markedly less nose scratching and sneezing, together with decreased eosinophilic upper-airway inflammation. Suzuki et al. studied the effects of CpG-ODN on established upper-airway inflammation in Japanese cedar pollen sensitized mice [24,35]. The mice that received intranasal therapy produced lower levels of IgE and IL-5 from nasal lymphocytes and splenocytes and exhibited less nasal eosinophilia and fewer signs of nasal irritation. Using a guinea pig model, Zhu et al. found that intranasal CpG-ODN administration significantly

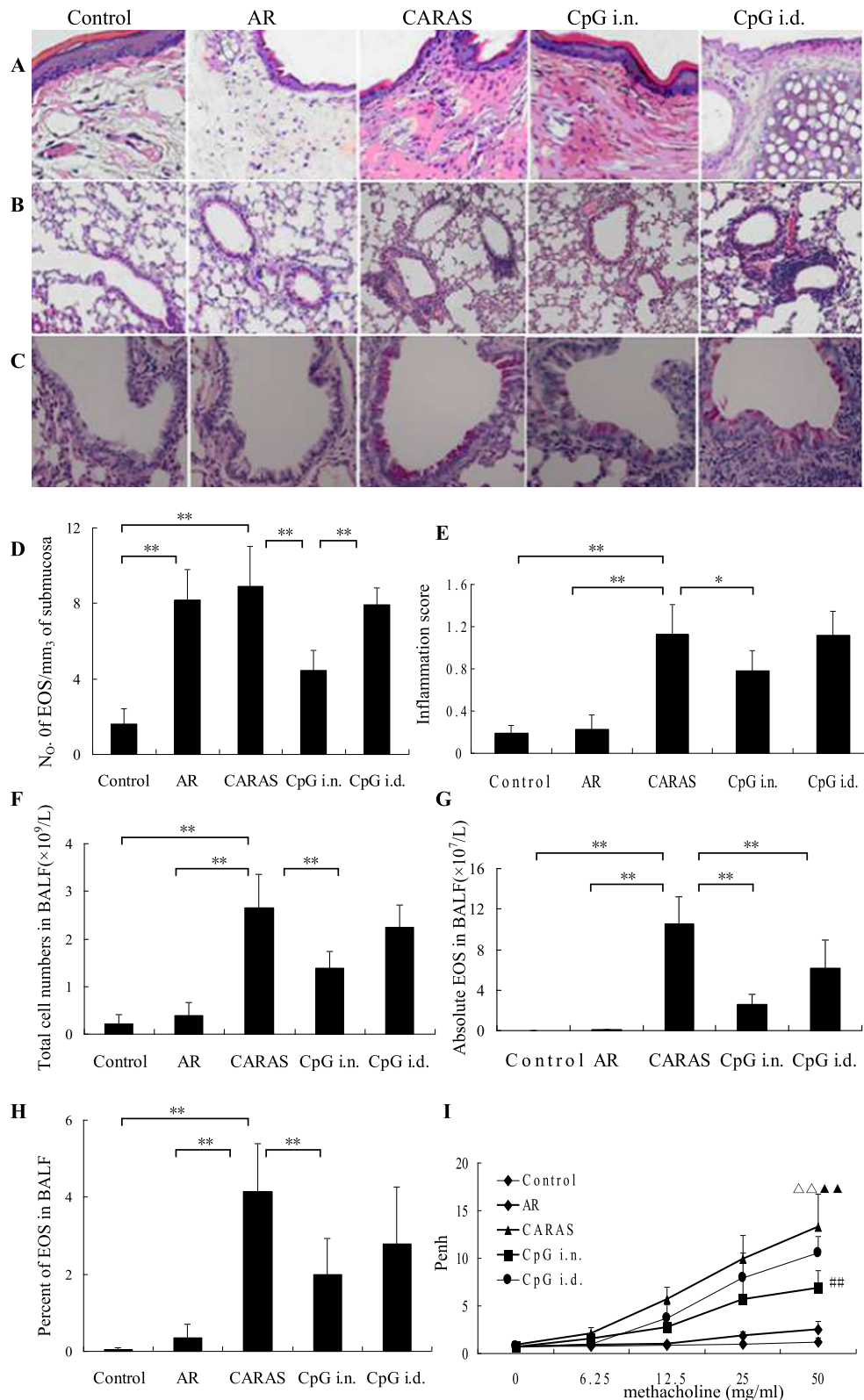


Fig. 4. Intranasal CpG-ODN therapy alleviates lower-airway cellular inflammation and reduces methacholine-induced AHR. Mice were sensitized and challenged as outlined in Fig. 1. The upper-airway submucosa and lower-airway inflammation were evaluated. Representative photomicrographs of H&E-stained nose (A) and lung sections (B), both original magnification, 10×. (C) Measurement of goblet cell metaplasia was estimated by PAS staining in lungs sections. Representative photographs are shown; original magnification, 40×. (D) The upper-airway submucosa eosinophilia was evaluated microscopically on the nasal septum. (E) The severity of peribronchial inflammation was analyzed using inflammation score as described in the text. The number of total cells (F), eosinophils (G) and the ratio of eosinophils to total cell numbers (H) in the BALF were analyzed under a light microscope as described in the text. AHR in response to inhaled methacholine was assessed by a mean of Penh (I). The data shown represent the means $\pm$ SD (n = 6 animals per group). * P < 0.05 or ** P < 0.01 compared with the counterpart groups. $\Delta\Delta P$ < 0.01 compared with the control group. $\Delta\Delta P$ < 0.01 compared with the AR group. $\Delta\Delta P$ < 0.01 compared with the CARAS group.

decreased the production of the OVA-specific Th2 cytokine IL-5 in both serum and nasal samples [36]. Recently, Campbell et al. developed a chronic allergic asthma model using ragweed-sensitized mice that were weekly intranasally exposed to ragweed. Using this model, the effects of a limited series of weekly intranasal 1018 ISS (CpG-ODN) treatments were evaluated during the treatment and for several weeks after the end of treatment; however, the weekly allergen exposures continued. The authors demonstrated that a short series of weekly CpG-ODN treatments inhibited ragweed-induced lung Th2 responses for several months, demonstrating the enduring suppression of allergic lung inflammation in the presence of frequent allergen exposures long after the completion of a limited CpG-ODN treatment course [37]. Comparable results were observed in previous studies of human AR subjects, which revealed that the treatment of peripheral blood mononuclear cells obtained from AR patients with CpG in vitro increased the IFN- γ levels, decreased the IL-4 levels, and suppressed IgE production [38]. Taken together, these results suggest that CpG-ODN therapy potentially reduces inflammation and shifts the immune response from a Th2 type toward a Th1-dominant type.

We initially hypothesized that CpG-ODN prevent asthma by inducing a Th1 response that counterbalances and suppresses the Th2 inflammation that is responsible for most asthmatic manifestations. In this study, however, we showed lower IL-4, IL-5 and IL-13 secretion from the BALF and the supernatants of cultured splenic lymphocytes after treatment with intranasal CpG-ODN relative to intradermal CpG-ODN treatment. However, intranasal CpG-ODN administration was not sufficiently potent to increase the levels of IFN- γ . Thus, our study indicates that intranasal CpG-ODN administration in vivo reduced the production of Th2 cytokines rather than increasing the production of Th1 cytokines. A recent report by Campbell et al. [37] also suggested that upon the establishment of a chronic ragweed-induced asthma model, persistent Th2 suppression was not reversible by IFN- γ neutralization. This finding demonstrates that IFN- γ is not required for maintaining CpG-ODN-induced disease modification. However, these data differ somewhat from the findings of other groups that found increased IFN- γ production due to CpG-ODN treatment [24,30]. Rhee et al. [39] reported that intradermal administration of CpG-ODN reduces Th2 responses in mice with AR. However, we were not able to demonstrate the effectiveness of the intradermal administration of CpG-ODN in mice with AR or CARAS. This discrepancy might be due to the following differences between our study and these other studies [24,39]: different murine strains were used between the aforementioned models; the mice received CpG-ODN before the nasal challenge in the study by Rhee et al. [39] but received CpG-ODN after the nasal challenge in our study; and different total amounts of CpG-ODN were administered (Rhee et al. used 100 μ g, whereas we used 10 μ g). It remains unclear why intradermal CpG-ODN administration does a decline of the IgE level, eosinophilia, and Th2 cytokines production by splenocytes or BALF from BALB/c mice treated according to the CARAS model, as shown in our study, but dose not significantly affect the aforementioned parameters (Figs. 2–4). One plausible explanation is that the inhibition of Th2 allergic responses by intradermal CpG-ODN is concentration-dependent [40]. CARAS might be more effectively attenuated by a higher concentration of intradermal CpG-ODN. However, the side effects of CpG-ODN at high doses should be considered [24,41]. Because the dose of intradermal administration CpG-ODN required for a protective effect was 10-fold greater than that of intranasal CpG-ODN administration in our CARAS mice and because the reduction in the IL-4 and IL-5 levels was notably smaller, alternate administration routes (e.g., intranasal CpG-ODN administration) warrant consideration.

We used the AR model described by KleinJan et al. [3], in which a compartmentalized allergic Th2 response is induced in the nose but not in the lower airways [42], to study the effects of upper and lower airway interactions on a lower-airway challenge. To induce upper-airway allergic inflammation, the mice were first sensitized via the peritoneal route and subsequently challenged by an intranasal instillation of either

OVA (into the AR mice) or PBS (into the control mice). Twenty-four hours after the final intranasal application of either OVA or PBS, the AR and control mice were challenged via exposure to with either aerosolized OVA or PBS induce lower-airway inflammation (see Fig. 1 for a time schedule). Many groups have studied nasal inflammation in murine models, but most of these groups used a large volume of nasal aspiration to challenge sensitized mice, leading to significant antigen deposition in the lungs and concomitant nasal and pulmonary inflammation, modeling CARAS [42]. To avoid antigen deposition in the lung, we repeatedly applied a 10- μ L droplet of OVA or PBS to the nose of awake mice over the course of 3 weeks [42]. As demonstrated in Fig. 4G–H, significantly higher percentages and absolute numbers of eosinophils were present in the BALF of the CARAS mice compared with the control mice. No eosinophilia was detected in the AR model (Fig. 4G–H). The levels of Th2 cytokines in the BALF were significantly higher in the CARAS mice than in the AR or control animals (Fig. 3, A and B). Based on these findings, we are convinced of the validity of our CARAS model for the study of the upper- and lower-airway interaction. Thus, the suppression of lower-airway inflammation that was observed in the mice that received the nasal instillation of CpG-ODN in the present study might have been due to targeted treatment of inflammation in the upper airways rather than pulmonary CpG-ODN deposition.

The role of CpG-ODN in treating allergic inflammation is currently an area of intense investigation. CpG-ODN resembles bacterial DNA and interacts with TLR-9 to exert multiple effects on the immune system, including the activation of DCs. These effects result in a cascade of immune responses that ultimately down-regulate the Th2 response, which can result in diverse manifestations of AR and asthma [40]. Based on the current understanding, CpG-ODN is extremely important contributors to Th1/Th2 cytokine homeostasis and might be an effective alternative to corticosteroids for the management of AR and asthma. Because almost all TLRs are positioned to play a sentinel role in the development of respiratory allergies, they provide major therapeutic targets to modulate the natural course of allergic disease [43]. Therefore, CpG-ODN holds considerable promise as a novel immunotherapeutic agent for the treatment of allergic diseases, especially CARAS. Common inflammatory processes appear to be the most important link in the cross-talk between the upper and lower airways. The nose and the lungs should be considered as one entity [11]. This important immunopathogenetic link between the upper and lower airways has implications for the approach to CARAS treatment. This understanding forms the rationale for using CpG-ODN to treat CARAS. Because AR and asthma are mediated by similar allergic inflammatory mechanisms, selective treatment of the nose could impact the lungs by reducing the systemic levels of proinflammatory cytokines, and the intranasal administration of CpG-ODN may therefore protect against the development of asthma. The TLR-9 gene [44] is up-regulated and TLR-9 is expressed in the nasal mucosa [45]. The effect of CpG-ODN on CARAS may involve the activation of TLR-9 in the nasal mucosa. In addition to their immunostimulatory activity, CpG-ODN display various drug-like characteristics, including high water solubility, ease of chemical synthesis on a large scale, and high stability and biodegradability [33,46]. Intranasal drug administration may enable rapid drug absorption and eliminate its liver peripheral removal without inducing systemic side effects [47]. In addition, the delivery of CpG-ODN via the nose is expected to be safe, convenient, and well tolerated [33] and protects the CpG-ODN from degradation by DNases [48]. Together with the results of other studies using animal models, our results provide a rationale for conducting clinical trials of CpG-ODN-based immunomodulation for the treatment of patients who suffer from both AR and asthma. Although further studies are necessary, our findings provide hope that new drugs, such as a CpG-ODN nasal spray, will be developed.

In summary, our data demonstrate that treatment with intranasal CpG-ODN suppressed nose and airway eosinophilia, decreased the systemic levels of OVA-specific IgE, reduced methacholine-induced AHR

and modestly decreased Th2-associated cytokine profiles in our CARAS model. Furthermore, intranasal CpG-ODN administration significantly relieved nasal symptoms. However, the aforementioned parameters were not significantly altered when CpG-ODN were administered intradermally. Our results indicate that CpG-ODN directly treat the upper airway and can improve lower-airway inflammation and AHR. The results of this study highlight the important role of intranasal CpG-ODN therapy in inhibiting the united airways allergic response by modulating of the homeostasis of the Th1/Th2 responses. These finding support the efforts to develop CpG-ODN as a novel therapeutic agent for CARAS.

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Competing interests

The authors have declared that no competing interests exist.

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