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To cite this article: Hong-tao Li, Zhuang-gui Chen, Hui Liu, Jin Ye, Xiao-ling Zou, Yan-hong Wang, Hai-ling Yang, Ping Meng & Tian-tuo Zhang (2016) Treatment of allergic rhinitis with CpG oligodeoxynucleotides alleviates the lower airway outcomes of combined allergic rhinitis and asthma syndrome via a mechanism that possibly involves in TSLP, *Experimental Lung Research*, 42:6, 322-333, DOI: [10.1080/01902148.2016.1215571](https://doi.org/10.1080/01902148.2016.1215571)

To link to this article: <http://dx.doi.org/10.1080/01902148.2016.1215571>

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ORIGINAL ARTICLE

Treatment of allergic rhinitis with CpG oligodeoxynucleotides alleviates the lower airway outcomes of combined allergic rhinitis and asthma syndrome via a mechanism that possibly involves in TSLP

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ABSTRACT

Purpose: Thymic stromal lymphopoietin (TSLP) is a critical regulator of immune responses associated with Th2 cytokine-mediated inflammation. Intranasal administration of oligodeoxynucleotides with CpG motifs (CpG-ODNs) might improve lower airway outcomes of combined allergic rhinitis and asthma syndrome (CARAS), but the inherent mechanisms of CpG-ODNs are not well defined. This study investigated whether CpG-ODNs treated to upper airway could reduce lower airway TSLP expression as well as whether this reduction could contribute to the alleviation of lower allergic inflammation and airway hyper-reactivity (AHR) in CARAS mice. **Materials and Methods:** Ovalbumin (OVA)-sensitized BALB/c mice were intranasal OVA exposure three times a week for 3 weeks. CpG-ODNs or an anti-TSLP mAb was administered to a subset of these mice 1 hour after intranasal OVA challenge, followed by 5 days of OVA aerosol challenge. The resulting immunological variables, nasal symptoms, and nasal mucosa and lung tissues pathology were evaluated. TSLP production in the lung tissues and bronchoalveolar lavage fluid (BALF) were determined by RT-PCR, western blotting or enzyme-linked immunosorbent assay. **Results:** The CARAS mice exhibited overexpression of TSLP in the lung tissues and BALF, and also demonstrated significant increases in BALF and splenocyte Th2-associated cytokine production, serum OVA-specific IgE, nose and lung pathologies, and AHR. Intranasal administration of CpG-ODNs restored TSLP in the lower airway, and it significantly reduced the following parameters: Th2-type cytokine production levels; the percentage of eosinophils in the BALF; IL-4 and IL-5 concentrations in the supernatants of cultured splenic lymphocytes; serum OVA-specific IgE; peribronchial inflammation score in the lungs; and nose pathology and nasal symptoms. Similar results were obtained when the CARAS mice were treated with an anti-TSLP mAb to block intranasal TSLP activity. **Conclusions:** Treatment with intranasal CpG-ODNs improves lower airway immunological variable outcomes in the CARAS model via a mechanism that possibly involves in suppressing pulmonary TSLP-triggered allergic inflammation.

ARTICLE HISTORY

Received 10 May 2016
Accepted 18 July 2016

KEYWORDS

asthma; allergic rhinitis; CpG oligodeoxynucleotides; cytokines; thymic stromal lymphopoietin

Introduction

Allergic rhinitis (AR) and asthma often coexist and are highly prevalent.^[1,2] This coexistence suggests the concept of “combined allergic rhinitis and asthma syndrome (CARAS),” “united airways” or “one airway-one disease”^[3–5] because an allergy is a systemic disease that causes symptoms in different target organs.^[6] AR is one of several risk factors for the development of asthma in both adults and children.^[7] One in three AR patients may progress to developing asthma within 10

years.^[8] AR can complicate asthma management and lead to poor asthma control and outcomes.^[2] Thus, the clinical signs of AR predate the development of asthma, suggesting that AR is an entry point for subsequent allergic diseases.

Because AR and asthma are inflammatory disorders that present 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

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toward Th1 has been of special interest to researchers in recent years.^[9,10] Previous studies have demonstrated that oligodeoxynucleotides (ODNs) with CpG motifs (CpG-ODNs) engage Toll-like receptor 9 (TLR-9) on dendritic cells (DCs), B-cells and other cell types as well as induce T helper (Th)1-type reactions^[11] and concomitantly inhibit Th2 responses.^[12] Recently, we reported that targeted treatment of the upper airway with intranasal CpG-ODNs reduce lower airway inflammation in a murine model of CARAS.^[13] However, the precise molecular mechanisms of CpG-ODNs that mediate interactions between the upper and lower airways are unknown.

Thymic stromal lymphopoietin (TSLP) is one of several epithelial-derived cytokines capable of promoting type-2 immune responses,^[14] which are associated with the pathogenesis of allergic inflammatory diseases, including AR and asthma.^[15] Compelling evidence demonstrates that TSLP represents one of the master switches initiating allergic inflammation and suggests that it has a determinant role in allergic diseases.^[16,17] We have previously reported that TSLP plays a pivotal role in the initiation and persistence of airway inflammation as well as remodeling in the context of chronic allergic asthma, thereby suggesting that appropriate inhibition of TSLP expression could be a novel therapeutic method for asthma.^[18] Other data further highlight the TSLP pathway as a relevant target in clinical human asthma.^[19] In a double-blind, placebo-controlled clinical study, Gauvreau et al. have found that treatment with AMG 157, a human anti-TSLP monoclonal immunoglobulin, reduced allergen-induced bronchoconstriction and indexes of airway inflammation in patients with mild allergic asthma.^[20] However, the existence of cross talk between CpG-ODNs and TSLP production in CARAS remains unclear. The link between CpG-ODNs and TSLP induction was first reported by Yamada et al.^[21] who shown that CpG-ODNs inhibit TSLP production in human atrytenoid fibroblasts. Considering that AR and asthma share a common underlying mechanism, AR may act as a starting point for CARAS. Therefore, it could be reasonable to speculate that treating AR with CpG-ODNs may improve lower airway inflammation in CARAS by down-regulating TSLP. To explore this hypothesis, we used our previously described murine OVA-driven model of CARAS,^[13] and we addressed this question by determining the expression levels of TSLP mRNA and protein involved in the intranasal

administration of CpG-ODNs during antigen challenge in CARAS mice. We focused on TSLP because its expression levels were significantly increased in asthmatic mice in our previous studies^[18,22] as compared to the levels of other genes.^[23] To the best of our knowledge, studies investigating the effects of CpG-ODNs and TSLP on allergic disorders are rare. The current results indicate that treatment of inflammation in the upper airways with CpG-ODNs improved the lower-airway inflammation and AHR in the CARAS model via a mechanism that might suppress pulmonary TSLP. Some results of this study were reported in the form of abstract.^[24]

Materials and methods

Mice

Female BALB/c mice (Sun Yat-sen University Animal Centre, China) were maintained under specific pathogen-free conditions in a temperature-controlled environment under a 12-hour light/dark cycle. At the onset of the experiments, all animals were between 6 and 8 weeks of age. All experiments were performed with the approval of the local Animal Ethics and Welfare Committee. To induce AR, the BALB/c mice were sensitized on days 0 and 7 by intraperitoneal injection with 10 µg of ovalbumin (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 OVA (1 mg/mL) three times a week for 3 weeks.^[5,13] Immediately after the last nasal challenge, the number of nasal rubbing movements and sneezing movements was recorded using a video recorder and counted for 30 minutes. Twenty-four hours after the last intranasal application of OVA, 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 for 5 consecutive days. For the CpG-ODNs experiment, the mice were intranasally administered 10 µg (1 mg/mL) of CpG-ODNs (Sangon Biotechnology, Shanghai, China), an amount that is known to be optimal for the stimulation of murine cells from a pilot study^[13] and another study,^[25] 1 hour after the nasal challenge (as indicated in [Figure 1A](#)). For the TSLP-blocking experiment, the mice received an intranasal administration of 20 µg (2 mg/mL) of anti-TSLP mAb (α TSLP Ab) (Abcam, USA) 1 hour prior to

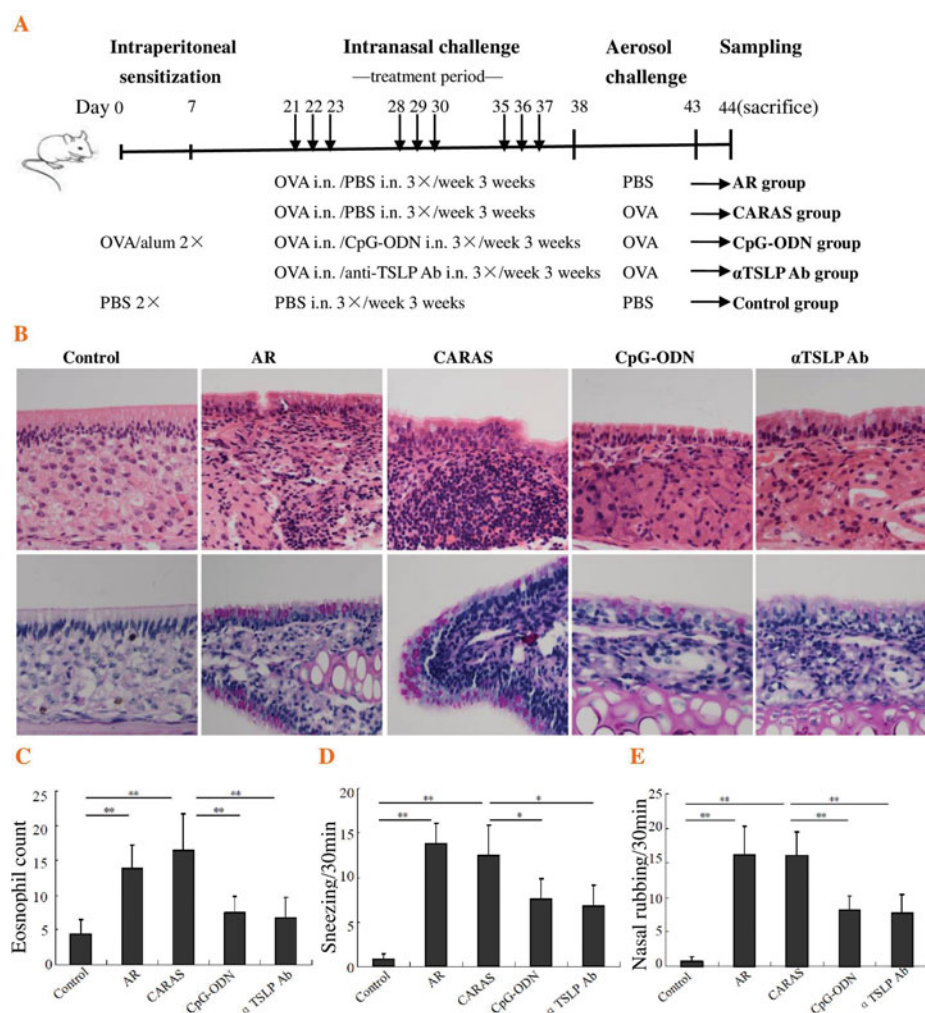


Figure 1. Intranasal CpG-ODNs treatment suppresses upper airway inflammation and nasal symptoms. (A) Protocol for generating the CARAS model. Mice were treated with intranasal CpG-ODNs or anti-TSLP mAb (α TSLP Ab) as described in Materials and Methods. (B) Histopathological analysis of mouse nasal mucosal tissues (images were taken at 10×40 magnification). Upper panels show H&E staining, and lower panels show PAS staining. (C) The number of eosinophils in the nasal septum was counted using a microscope at 10×40 magnification. (D and E) Nasal symptoms were presented as sneezing and scratching of the nose. Frequency of sneezing and scratching was monitored for 30 minutes after last intranasal challenge. Data are presented as the mean $\pm$ SD, and the difference between groups was significant ($P < .05$) according to analysis of variance (ANOVA). * $P < .05$ or ** $P < .01$ compared with the counterpart groups.

OVA administration (Figure 1A). The anti-TSLP mAb dose was predetermined by a staining analysis of airway inflammation in mice that received 10–40 μ g of anti-TSLP mAb as previously described.^[18] The control mice were treated with PBS in the same way as the experimental mice, except that the OVA, CpG-ODNs, and anti-TSLP mAb were omitted during both the sensitization and challenge phases (Figure 1A).

Assessment of airway reactivity

Airway resistance was measured using a methacholine-induced airflow obstruction with a whole-body plethysmograph (Buxco Electronics, USA) according to the methods described in our previous report.^[13,18]

Histological examination and assessment

Nasal tissues were isolated using previously published methods with a slight modification.^[12,26] When mice were painlessly sacrificed by cervical dislocation, nasal septum mucosal tissue from six mice in each group was fixed with paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) to count eosinophil and goblet cells, respectively. The pathomorphology of the mouse nasal mucosa was observed under an optical microscope. The number of eosinophils in the submucosal region of the nasal septum was quantified under a microscope in five fields at $40\times$ magnification in a blinded manner.^[27]

Lung tissues were isolated according to our previously published methods.^[13,18] The lungs were dissected from the chest cavity after lavage. The left lung was immediately fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned (5- μ m slices). To assess lower airway inflammation, the sections were stained with H&E or PAS. The severity of peribronchial inflammation was scored by a blinded observer according to the previously described features.^[28]

Bronchoalveolar lavage fluid (BALF) and cell collection

Bronchoalveolar lavage fluid (BALF) was obtained by the infusion and collection of 0.5 mL of saline. The BALF was centrifuged at 2000 rpm for 10 min at 4°C, and the supernatant was stored at -80°C for further cytokine quantification by enzyme-linked immunosorbent assay (ELISA). The differential cell counts in the BALF were performed as described in our previous report.^[13,18]

Enzyme-linked immunosorbent assay (ELISA) for cytokines

The concentrations of TSLP, IL-4, IL-5, IL-13, and IFN- γ in the BALF were measured using ELISA kits (TSLP: Abcam, USA; IL-4, IL-5, IL-13, and IFN- γ : Elabsciences, Wuhan, China) according to the manufacturer's instructions. A total of 0.7 mL of blood from each mouse was collected by cardiac exsanguination. The serum levels of OVA-specific IgE were determined using an ELISA assay (Elabsciences, Wuhan, China). The serum total IgE levels were not detected due to the negative results in our previous study.^[13]

RNA extraction and real-time quantitative PCR analysis

Total RNA in the right lung samples was isolated with Trizol (Invitrogen) as described in our previous report with slight modification.^[18] The RNA was quantified by measuring the absorption at 260 nm, and the purity of the RNA was assayed by the ratio of absorbance at 260 nm and 280 nm. A fraction was electrophoresed with 1% agarose to verify its integrity. The total RNA of each sample was reverse transcribed into cDNA. Real-time PCR was performed to measure the mRNA levels of TSLP, IL-4, IL-5, IL-13, and IFN- γ using the primers

Table 1. Real-time PCR primer sequences.

Target gene	Direction	Sequence
TSLP	Forward primer	5'-ACGGATGGGGCTAAGTACAA-3'
	Reverse primer	5'-AGTCCTCGATTGCTCGAACT-3'
GAPDH	Forward primer	5'-AGCTTCGGCAGATATTCATCTG-3'
	Reverse primer	5'-CGTTCACTCCCATGACAAACA-3'
IL-4	Forward primer	5'-CCATATCCACGGATGCGACA-3'
	Reverse primer	5'-CGTTGCTGTGAGGACGTTTG-3'
IL-5	Forward primer	5'-TGAGGCTTCTGTCCCTACT-3'
	Reverse primer	5'-CCCCACGGACAGTTTGATT-3'
IL-13	Forward primer	5'-ACCGAAATGTTGATAGCGACAG-3'
	Reverse primer	5'-ACAATGCTCTGACAAATGCGTA-3'
IFN- γ	Forward primer	5'-CAGCAACAGCAAGGCGAAAA-3'
	Reverse primer	5'-TCATTGAATGCTTGCGCTG-3'

described in Table 1. Using GAPDH as a housekeeping gene, the mRNA was analyzed with a real-time PCR system (AB7500, Applied Biosystems, Foster City, CA, USA) using the following conditions: 2 minutes at 94°C followed by 40 cycles of 20 seconds at 94°C, 20 seconds at 58°C, and 20 seconds at 72°C.

Western blot analysis

Western blot analysis was performed as previously described.^[29] Briefly, the lung tissues lysate was centrifuged to obtain the supernatant. The total protein concentration of the supernatant was measured by a BCA Protein Assay Kit (Pierce, USA). The lung cell lysate proteins were separated on sodium dodecyl sulphate polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Merck Millipore, USA). The membranes were incubated with primary antibodies against TSLP (Abcam, USA) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Abcam, USA; used as the internal reference control) overnight at 4°C. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (Boster, China) for 1 hour. The signals were detected using Image-Pro Plus 6.0 Analyzer software (Media Cybernetics, USA).

In vitro splenic cell restimulation with OVA

In our previous report, we demonstrated that CpG-ODNs suppress Th2-type cytokines in BALF. To clarify whether CpG-ODNs can modulate the Th1/Th2 cytokine profile systemically, we performed a recall antigen response assay as our previously described.^[13] Lymphoid cells (4×10^5 cells per 200 μ L) from spleen were prepared after mice were sacrificed. The cells were stimulated with 10 μ g/mL OVA at 37°C in

an atmosphere of 5% CO₂ for 4 days as previously described.^[5,12,13] After the incubation, the cell-free culture supernatants were harvested and stored for Th2-type cytokine quantification using commercially available ELISA kits in accordance with the manufacturer's instructions (Elabsciences, China).

Data analysis

The STATA 10.0 statistical software package (Stata Corp LP) was used for the analyses. All results are presented as the means $\pm$ SD. For statistical evaluations, one-way ANOVA was used to calculate the overall *P*-value, and Bonferroni's multiple comparison of means was used as a posttest. A *P*-value of $< .05$ was considered a significant difference between groups.

Results

Treatment of upper airway with intranasal CpG-ODNs suppresses upper airway inflammation and nasal symptoms

We first assessed the distribution and severity of allergen-induced intranasal inflammation and observed that the OVA intranasal challenge induced nasal inflammation in the sensitized mice (Figure 1B). The mice with AR and CARAS developed significant submucosal eosinophilia compared with control mice (both $P < .01$, Figure 1C). Intranasal CpG-ODNs therapy and blockade of TSLP activity with an anti-TSLP mAb resulted in fewer eosinophils than in untreated CARAS mice (both $P < .01$, Figure 1C). Furthermore, AR and CARAS exhibited goblet cell hyperplasia in the nasal mucosa of mice, which was inhibited by the CpG-ODNs or anti-TSLP mAb (Figure 1B). To examine the effects of CpG-ODNs on nasal symptomatology, we counted the number of nasal rubbings and sneezing movements for 30 minutes immediately after the last nasal challenge. Mice in the control group were as quiet as before the mock treatment, and no obvious behavioral abnormalities were observed. Compared to the control mice, the animals with AR and CARAS demonstrated significant increases in nasal rubbing and sneezing (both $P < .01$, Figure 1D and 1E). The number of nasal symptoms was significantly lower in mice with CARAS that received intranasal CpG-ODNs or anti-TSLP mAb therapy than in mice that received no therapy (both $P < .05$, Figure 1D and 1E). These

findings were consistent with the changes in nasal inflammation following CpG-ODNs or anti-TSLP mAb treatment.

Intranasal CpG-ODNs therapy inhibits the levels of markers of both lower airway inflammation and airway hyper-responsiveness

The aforementioned data clearly demonstrated that the administration of intranasal CpG-ODNs suppresses upper airway inflammation and nasal symptoms. Because AR predates the development of asthma,^[7] we further analyzed the levels of Th1/Th2-associated cytokines in the BALF and lung tissues of the experimental animals to elucidate the contribution of CpG-ODNs treatment to Th1/Th2 cytokine homeostasis in the lower airway of the CARAS model. As shown in Figure 2A and 2B, the levels of IL-4, IL-5, and IL-13 (both protein and mRNA) were higher in the CARAS mice than in the control animals or in the AR animals (both $P < .05$). CpG-ODNs or anti-TSLP mAb intranasal treatment significantly reduced the levels of IL-4, IL-5, and IL-13 (both protein and mRNA) compared to the levels found in the CARAS mice (both $P < .05$, Figure 2A and 2B). Furthermore, the CARAS mice exhibited a significant shift in the IFN- γ /IL-4 ratio compared with the AR mice ($P < .05$, Figure 2C). However, this change was significantly reversed by intranasal CpG-ODNs or anti-TSLP mAb treatment (both $P < .01$, Figure 2C). Consistently, significantly higher levels of OVA-induced Th2-type cytokine (IL-4 and IL-5) production were observed in the spleen cells obtained from the mice with AR and CARAS compared with control mice (both $P < .05$, Figure 2D). Splenocytes and lymphocytes in BALB/c mice that had received intranasal CpG-ODNs or anti-TSLP mAb therapy released significantly lower levels of IL-4 and IL-5 than the BALB/c mice that received no therapy (both $P < .05$, Figure 2D).

To determine if the protective effects of CpG-ODNs previously observed in the AR model^[12] would further inhibit lower airway cellular inflammation after repeated exposure to aerosolic OVA, we examined the severity of lower airway inflammation using the peribronchial inflammation score.^[28] We found that animals with AR exposed to OVA for 5 consecutive days (i.e., CARAS mice) exhibited robust airway inflammation that was characterized by the influx of inflammatory cells, including eosinophils and lymphocytes

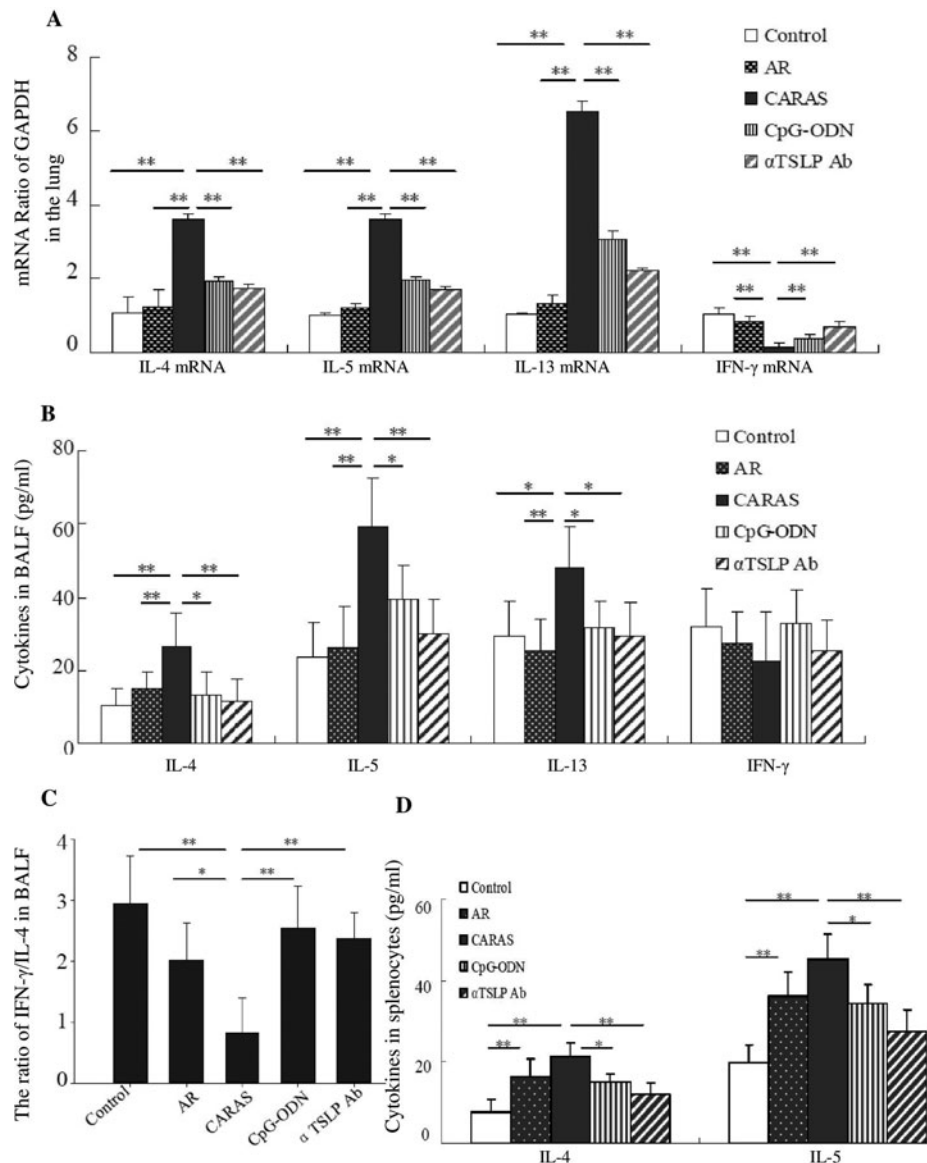


Figure 2. Intranasal CpG-ODNs therapy alters Th2-type responses from lower airway and splenocytes. (A) Relative mRNA expression of Th1/Th2 cytokines in lung tissues of mice. mRNA expression was analyzed by real-time PCR. (B) ELISA of Th1/Th2-associated cytokine levels in the BALF. (C) The IFN- γ /IL-4 ratio in the BALF was evaluated by ELISA. (D) Th2-type cytokine production levels in culture supernatants of splenocytes were determined by ELISA. Each bar in the diagrams represents the mean $\pm$ SD of six independent experiments. * $P < .05$ or ** $P < .01$ compared with the counterpart groups.

(Supplementary Figure S1A). The inflammation score of the CARAS mice was significantly higher than that of AR mice and control animals (both $P < .01$, Supplementary Figure S1B) and was significantly lower than that of mice treated intranasally with CpG-ODNs or anti-TSLP mAb (both $P < .05$, Supplementary Figure S1B). The total cell number, the absolute number of eosinophils, and the percentage of eosinophils were higher in the BALF of CARAS mice than in that of PBS-treated controls and the AR group (all $P < .01$, Supplementary Figure S1C-E). CpG-ODNs or anti-TSLP mAb intranasal therapy markedly inhibited

the observed values in CARAS mice (all $P < .05$, Supplementary Figure S1C-E), except for the percentage of lymphocytes (both $P > .05$, Supplementary Figure S1F). Because goblet cell hyperplasia is a common feature of asthmatic airways, we next evaluated airway goblet cells and mucus with PAS staining. The CARAS mice showed significant luminal mucus hypersecretion and goblet cell hyperplasia in the airway epithelia compared to the PBS-treated control mice (Supplementary Figure S1A and S1G). Pretreatment with either CpG-ODNs or anti-TSLP mAb markedly inhibited mucus hyper-secretion and goblet cell hyperplasia

(both $P < .01$, Supplementary Figure S1A and S1G). In addition, the CARAS mice subjected to intranasal CpG-ODNs or anti-TSLP mAb therapy exhibited significantly lower levels of OVA-specific IgE than the CARAS mice receiving no therapy (both $P < .01$, Supplementary Figure S2A). Furthermore, the CARAS mice exhibited significantly greater responses and sensitivity to inhaled methacholine than the PBS-treated control group and the AR group, while treatment with intranasal CpG-ODNs or anti-TSLP mAb significantly decreased AHR (both $P < .05$, Supplementary Figure S2B). However, CpG-ODNs treatment did not demonstrated a superior effect on the AHR compared with anti-TSLP mAb treatment ($P > .05$, Supplementary Figure S2B). These findings indicated that a robust Th2 response was induced in the CARAS mice. Moreover, intranasal CpG-ODNs treatment markedly suppressed the dominant production of Th2-type immune responses and resulted in lower airway inflammation and methacholine-induced AHR in the CARAS mice. Similar results were obtained after anti-TSLP therapy with the anti-TSLP mAb. Therefore, these data suggested that the effects of CpG-ODNs treatment on markers of lower airway inflammation and AHR might be mediated by the downregulation of TSLP.

Intranasal CpG-ODNs therapy inhibits TSLP mRNA and protein expression in the lower airway of CARAS mice

To determine if intranasal administration CpG-ODNs could reduce lower airway TSLP levels, which is pivotal for the generation of Th2-type inflammation,^[30,31] we measured TSLP mRNA expression in lung tissues of mice using quantitative real time PCR. In CARAS mice, lung TSLP mRNA expression was significantly increased compared to TSLP levels in AR and control mice (both $P < .01$, Figure 3A). Furthermore, ELISA indicated that the TSLP protein levels in the BALF of CARAS mice were significantly increased compared to those of AR mice and control mice (both $P < .05$, Figure 3B). Using Western blotting, we detected higher TSLP protein expression in the lung tissues of CARAS mice compared to AR and control mice (both $P < .01$, Figure 3C). Because TSLP production can be induced by TLR ligands,^[32] we examined the effects of CpG-ODNs (TLR9 agonist) and anti-TSLP mAb upper airway-targeted treatments on lower airway TSLP production in CARAS mice. Lung TSLP mRNA expression was significantly reduced in treated CARAS mice compared to untreated CARAS mice (both $P < .01$, Figure 3A). Moreover, TSLP protein

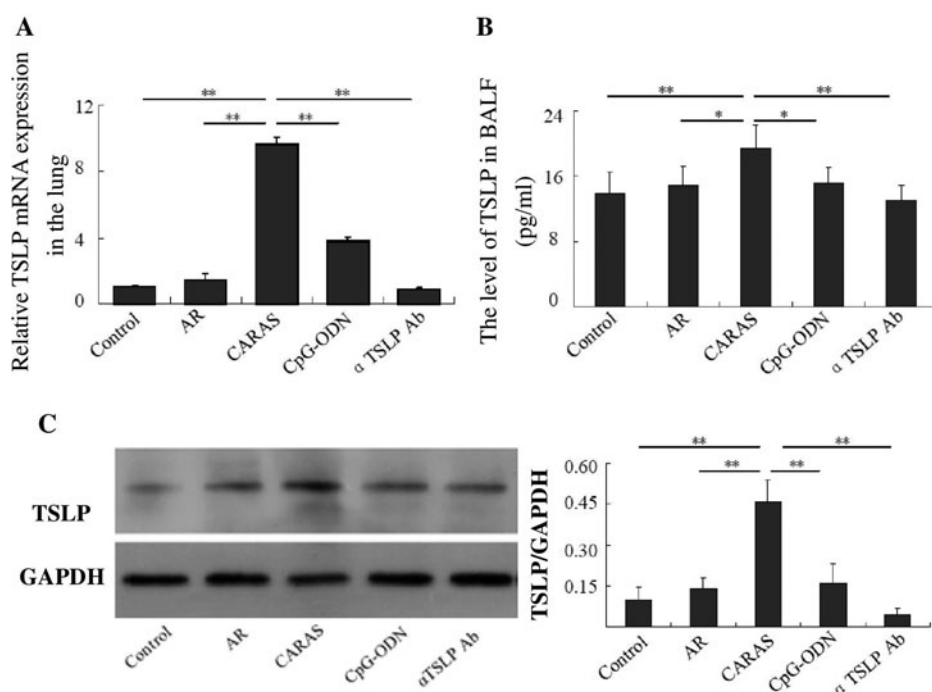


Figure 3. Intranasal CpG-ODNs therapy inhibits TSLP expression in the lower airway of CARAS mice. (A) Relative TSLP mRNA levels in lung were detected using real-time PCR. (B) TSLP protein concentrations in BALF were detected using an ELISA kit. (C) Western blot results for TSLP in the lung tissues of mice. GAPDH was used as an internal control. The data shown represent the means $\pm$ SD ($n = 6$ animals per group). * $P < .05$ or ** $P < .01$ compared with counterpart groups.

levels in the lung cell lysates were also significantly reduced by threefold to fourfold in CpG-ODNs- or anti-TSLP mAb-treated CARAS mice compared to untreated CARAS mice (both $P < .05$, Figure 3C) as determined by western blotting (Figure 3C). Furthermore, the ELISA demonstrated that TSLP protein levels in the BALF of CARAS mice were significantly reduced when the CARAS mice were subjected to intranasal CpG-ODNs or anti-TSLP mAb treatment (both $P < .05$, Figure 3B). Taken together, these results demonstrated that targeted treatment of nasal mucosal tissue in the upper airway with CpG-ODNs suppresses TSLP mRNA and protein levels in the lower airway of CARAS mice. Comparable results were observed when TSLP activity was neutralized with an anti-TSLP mAb.

Discussion

Individuals with one atopic disease are far more likely to develop a second disease.^[33] The hypothesis of the “atopic march” has gained popularity in recent years. AR could act as the starting point of the atopic march.^[34] Murine models have identified TSLP as a key molecule for atopic march progression.^[33,35] The “hygiene hypothesis,” which was combined with known features of the atopic march,^[36] links an increased risk of atopic disease with reduced microbial exposure during childhood. Toll-like receptors (TLRs) are found on the cell surface and within the cell, where they facilitate the recognition of and response to microbes and their components. CpG-ODNs resemble bacterial DNA and act as TLR9 agonists. Previous studies have examined the effects of CpG-ODNs on allergic inflammation. Mice treated with CpG-ODNs exhibit markedly less nasal scratching and sneezing in addition to decreased eosinophilic upper airway inflammation.^[37] Our previous study also demonstrated that intranasal administration of CpG-ODNs significantly relieves lower-airway inflammation and AHR in CARAS mice.^[13] Taken together, these results lend support to the unified airway theory, suggesting that upper airway treatment can affect lower airway inflammation. Intranasal administration of CpG-ODNs might be a novel therapeutic strategy for the treatment of CARAS. However, the molecular and cellular pathways that cause the effects of CpG-ODNs treatment on the interaction between the upper and lower airways remain unexplored. Because TSLP can be induced by exogenous stimuli, such as TLR ligands,^[38] we hypothesized that

the effects of CpG-ODNs (TLR 9 agonist) on the nose-lung interaction might be mediated through TSLP.

Our results suggested that the OVA-sensitized mice, which were subjected to three weeks of continuous intranasal OVA exposure followed by 5 days of OVA aerosol challenge (i.e., CARAS mice), not only had local upper airway nasal eosinophilia inflammation and goblet cell hyperplasia (Figure 1) but also had Th2-mediated lower airway lung inflammation. The inflammation was demonstrated by dramatic increases in the levels of IL-4, IL-5, and IL-13 in the lower airway (both protein and RNA levels) as well as by BAL cellularity, lung pathology, increased serum OVA-specific IgE levels, and methacholine-induced AHR (Figure 2, Supplementary Figures S1 and S2). In addition, the CARAS mice exhibited overproduction of TSLP in lung tissues and BALF (Figure 3). Although used only in the upper airway, intranasal treatment with CpG-ODNs markedly suppressed not only the upper airway nasal inflammation and nasal symptoms (Figure 1) but also the dominant production of Th2-type immune responses, lower airway inflammation and AHR in the CARAS mouse model (Figure 2, Supplementary Figures S1 and S2). Furthermore, CpG-ODNs treatment suppressed TSLP mRNA and protein expression in the lower airway of CARAS mice (Figure 3). Similar results were obtained when the CARAS mice were subjected to intranasal blockade of TSLP activity using an anti-TSLP mAb (Figures 2 and 3, Supplementary Figures S1 and S2). Because CpG-ODNs treatment suppressed the levels of TSLP expression in the lower airway of CARAS mice with comparable results to the use of intranasal administration of a TSLP-neutralizing antibody, we, therefore, think that the suppression of pulmonary TSLP might participate in the effects of CpG-ODNs on lower airway inflammation.

However, it is necessary to point out that our present data could not exclude completely the possibility that CpG-ODNs reduced TSLP production indirectly by feedback of the inhibition of CpG-ODNs on CARAS via a mechanism that may involve CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs), which are known to suppress the activity of effector T lymphocytes^[39] by releasing anti-inflammatory cytokines such as IL-10.^[40,41] Asthma patients are known to have fewer and less functional Tregs.^[42–44] CpG-ODNs have been shown to modulate Th2 inflammation in the lung by induction of Tregs in a cockroach-induced asthma model,^[45] and increasing CD4⁺Foxp3⁺ Tregs

might suppress TSLP and Th17 responses in the airways.^[39] IL-25 may affect Tregs activity^[46] and promote and augment Th2 responses locally or systemically. Although growing evidence from experimental and clinical studies indicates that the expression of IL-25 is markedly upregulated in asthmatic conditions,^[47] the details underpinning how IL-25 orchestrates type 2 immunity have only been uncovered.^[48] IL-25 may activate mDCs to initiate type 2 immune responses in the airway, and also prime pDCs to enhance host defense against airway virus infection, as evidenced by pDCs stimulated *in vitro* with IL-25 increase expression of TLR9 and show heightened activation responses to TLR9 agonists.^[49] Both IL-25 and TSLP can induce production of Th2-type cytokines; however, the relationships between TSLP and IL-25 still require further investigation.^[48] Because Tregs and IL-25 are known to regulate aberrant immune responses, such as those that occur in asthma, it is valuable if we investigate a role for Tregs and IL-25 in CpG-ODNs-mediated suppression of OVA-induced CARAS, which might be a priority for our future research.

To avoid nasal instillation of CpG-ODNs or anti-TSLP mAb deposition in the lung, we repeatedly applied a small volume (10- μ L) droplet of OVA, PBS to the nose of awake mice over the course of 3 weeks as described in our previous study^[13] and Klein-Jan et al.^[5] In contrast, many study groups used a large volume of nasal aspiration to challenge sensitized mice, which may lead to significant antigen deposition in the lungs and concomitant nasal and pulmonary inflammation.^[50] As demonstrated in Figure S1, significantly higher percentages and absolute numbers of eosinophils were present in the BALF of the CARAS mice compared with the control mice. Few eosinophilias were detected in the AR model (Supplementary Figure S1). The levels of Th2 cytokines in the BALF were significantly higher in the CARAS mice than in the AR or control animals (Figure 2). Based on these findings, we are convinced of the validity of the 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-ODNs in the present study might have been due to treatment of inflammation in the upper airways rather than pulmonary CpG-ODNs deposition.

Based on the knowledge gained thus far, CpG-ODNs are important contributors to Th1/Th2 cytokine

homeostasis and might be an effective alternative to corticosteroids for the management of AR and asthma. CpG-ODNs involving TLR 9 hold considerable promise as a novel immunotherapeutic agent for the treatment of allergic diseases, especially CARAS. Naturally, certain limitations must also be considered. Differences in the immune responses between mice and humans demand care when extrapolations are made. Although OVA is the most widely studied allergen, it is not the strongest Th2 inducer and lacks the intrinsic enzymatic (e.g., protease) activities possessed by most natural allergens.^[36,51] However, together with the results of other studies, our results provide a rationale for conducting clinical trials using CpG-ODN-based immunomodulation for the treatment of patients who suffer from both AR and asthma. CpG-ODNs have been currently used in clinical trials. It was recently reported that treatment with CYT003, which is a TLR-9 agonist immunomodulator and consists of a bacteriophage capsid filled with an CpG, significantly improved patient reported measures and change in FEV₁ in patients with mild-to-moderate persistent allergic asthma in a Phase 2a clinical study,^[52] but CYT003 is ineffective as an add-on therapy to ICS plus LABA in patients with moderate-to-severe allergic asthma in a Phase 2b study.^[53] The reasons for apparent discrepancy between Phases 2a and 2b study could relate to differences in disease severity and experimental design (mild and well-controlled asthma with undergoing controlled steroid withdrawal versus moderate-to-severe asthma with ICS/LABA therapy). Indeed, patients with insufficiently controlled moderate-to-severe asthma receiving medium-to-high doses of ICS pose a potentially huge hurdle in clinical trials, as there may only be a narrow window for further improvements. Therefore, the results of the Phase 2b study should not preclude future studies from targeting TLR-9 in allergic asthma, as this approach is based on convincing scientific rationale.^[47]

In conclusion, our present study suggested that the direct treatment of the upper airway with CpG-ODNs improves lower airway inflammation and AHR possibly involves in restoring the overexpression of pulmonary TSLP. The results of our study elucidated a novel mechanism for CpG-ODNs treatment and implicated CpG-ODNs as novel therapeutic agents for CARAS. AR and asthma are now increasingly being diagnostically and therapeutically approached via the "one airway concept." Optimal treatment consists of a

combined strategy to treat the upper and lower airways, thus ensuring an acceptable efficacy/safety ratio. Therefore, future clinical studies for the CpG-ODNs treatment of patients with coexisting conditions might be warranted based on these data.

Acknowledgments

The authors thank Li-xian Yin and Min Dong for technical assistance. This manuscript was edited for English language by American Journal Experts (AJE).

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Funding

This study was supported by the National Natural Science Foundation of China (grant numbers: 81470220, 81370114, and 81470219) and the Guangdong Natural Science Foundation (grant number: S2013010015990).

References

- [1] de Groot EP, Nijkamp A, Duiverman EJ, Brand PL. Allergic rhinitis is associated with poor asthma control in children with asthma. *Thorax*. 2012;67:582–587.
- [2] Lohia S, Schlosser RJ, Soler ZM. Impact of intranasal corticosteroids on asthma outcomes in allergic rhinitis: a meta-analysis. *Allergy*. 2013;68:569–579.
- [3] Corren J. The connection between allergic rhinitis and bronchial asthma. *Curr Opin Pulmon Med*. 2007;13:13–18.
- [4] Taramarcas P, Gibson PG. The effectiveness of intranasal corticosteroids in combined allergic rhinitis and asthma syndrome. *Clin Exp Allergy*. 2004;34:1883–1889.
- [5] KleinJan A, Willart M, van Nimwegen M, Leman K, Hoogsteden HC, Hendriks RW, et al. United airways: circulating Th2 effector cells in an allergic rhinitis model are responsible for promoting lower airways inflammation. *Clin Exp Allergy*. 2010;40:494–504.
- [6] Valovirta E. Managing co-morbid asthma with allergic rhinitis: targeting the one-airway with leukotriene receptor antagonists. *World Allergy Organ J*. 2012;5:S210–S211.
- [7] Guerra S, Sherrill DL, Martinez FD, Barbee RA. Rhinitis as an independent risk factor for adult-onset asthma. *J Allergy Clin Immunol*. 2002;109:419–425.
- [8] Bousquet J, Van Cauwenberge P, Khaltaev N. Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol*. 2001;108:S147–S334.
- [9] Heeg K, Dalpke A, Peter M, Zimmermann S. Structural requirements for uptake and recognition of CpG oligonucleotides. *Int J Med Microbiol*. 2008;298:33–38.
- [10] Jurk M, Vollmer J. Therapeutic applications of synthetic CpG oligodeoxynucleotides as TLR9 agonists for immune modulation. *BioDrugs*. 2007;21:387–401.
- [11] Marshall JD, Fearon K, Abbate C, Subramanian S, Yee P, Gregorio J, et al. Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. *J Leukoc Biol*. 2003;73:781–792.
- [12] Suzuki M, Matsumoto T, Ohta N, Min WP, Murakami S. Intranasal CpG DNA therapy during allergen exposure in allergic rhinitis. *Otolaryngol Head Neck Surg*. 2007;136:246–251.
- [13] Li HT, Zhang TT, Chen ZG, Ye J, Liu H, Zou XL, et al. Intranasal administration of CpG oligodeoxynucleotides reduces lower airway inflammation in a murine model of combined allergic rhinitis and asthma syndrome. *Int Immunopharmacol*. 2015;28:390–398.
- [14] Saenz SA, Taylor BC, Artis D. Welcome to the neighborhood: epithelial cell-derived cytokines license innate and adaptive immune responses at mucosal sites. *Immunol Rev*. 2008;226:172–190.
- [15] Zhang Y, Zhou B. Functions of thymic stromal lymphopoietin in immunity and disease. *Immunol Res*. 2012;52:211–223.
- [16] Larson RP, Zimmerli SC, Comeau MR, Itano A, Omori M, Iseki M, et al. Dibutyl phthalate-induced thymic stromal lymphopoietin is required for Th2 contact hypersensitivity responses. *J Immunol*. 2010;184:2974–2984.
- [17] Li YL, Li HJ, Ji F, Zhang X, Wang R, Hao JQ, et al. Thymic stromal lymphopoietin promotes lung inflammation through activation of dendritic cells. *J Asthma*. 2010;47:117–123.
- [18] Chen ZG, Zhang TT, Li HT, Chen FH, Zou XL, Ji JZ, et al. Neutralization of TSLP inhibits airway remodeling in a murine model of allergic asthma induced by chronic exposure to house dust mite. *PLoS One*. 2013;8:e51268.
- [19] Froidure A, Shen C, Gras D, Van Snick J, Chanez P, Pilette C. Myeloid dendritic cells are primed in allergic asthma for thymic stromal lymphopoietin-mediated induction of Th2 and Th9 responses. *Allergy*. 2014;69:1068–1076.
- [20] Gauvreau GM, O'Byrne PM, Boulet LP, Wang Y, Cockcroft D, Bigler J, et al. Effects of an anti-TSLP antibody on allergen-induced asthmatic responses. *N Engl J Med*. 2014;370:2102–2110.
- [21] Yamada T, Saito H, Kimura Y, Kubo S, Sakashita M, Susuki D, et al. CpG-DNA suppresses poly(I:C)-induced TSLP production in human laryngeal arytenoid fibroblasts. *Cytokine*. 2012;57:245–250.
- [22] Li HT, Zhang TT, Chen ZG, Huang J, Liu H, Zhu JX. The effect of budesonide on thymic stromal lymphopoietin receptor and cytokine profile of dendritic cells in OVA-induced asthma in mice. *Chin J Tuberc Respir Dis*. 2012;35:497–502.

- [23] Ashrin MN, Arakaki R, Yamada A, Kondo T, Kurosawa M, Kudo Y, et al. A critical role for thymic stromal lymphopoietin in nickel-induced allergy in mice. *J Immunol.* 2014;192:4025–4031.
- [24] Li HT, Chen ZG, Liu H, Ye J, Zou XL, Wang YH, et al. Targeted treatment allergic rhinitis with CpG oligodeoxynucleotides reduces lower airway inflammation of combined allergic rhinitis and asthma syndrome via down-regulation thymic stromal lymphopoietin. Presented at the American Thoracic Society 2016 international scientific conferences. May 13–18, 2016, San Francisco, USA. <https://cms.psav.com/cPaper2016/ats2016>
- [25] Chen L, Arora M, Yarlalagadda M, Oriss TB, Krishnamoorthy N, Ray A, et al. Distinct responses of lung and spleen dendritic cells to the TLR9 agonist CpG ligodeoxynucleotide. *J Immunol.* 2006;177:2373–2383.
- [26] Miyata M, Hatsushika K, Ando T, Shimokawa N, Ohnuma Y, Katoh R, et al. Mast cell regulation of epithelial TSLP expression plays an important role in the development of allergic rhinitis. *Eur J Immunol.* 2008;38:1487–1492.
- [27] Ren J, Deng Y, Xiao B, Wang G, Tao Z. Protective effects of exogenous surfactant protein A in allergic rhinitis: a mouse model. *Ann Otol Rhinol Laryngol.* 2013;122:240–246.
- [28] Nakagami Y, Favoreto S Jr, Zhen G, Park SW, Nguyenvu LT, Kuperman DA, et al. The epithelial anion transporter pendrin is induced by allergy and rhinovirus infection, regulates airway surfaceliquid, and increases airway reactivity and inflammation in an asthma model. *J Immunol.* 2008;181:2203–2210.
- [29] Deng R, Su Z, Lu F, Zhang L, Lin J, Zhang X, et al. A potential link between bacterial pathogens and allergic conjunctivitis by dendritic cells. *Exp Eye Res.* 2014;120:118–126.
- [30] Mou Z, Xia J, Tan Y, Wang X, Zhang Y, Zhou B, et al. Overexpression of thymic stromal lymphopoietin in allergic rhinitis. *Acta Otolaryngol.* 2009;129:297–301.
- [31] Kimura S, Pawankar R, Mori S, Nonaka M, Masuno S, Yagi T, et al. Increased expression and role of thymic stromal lymphopoietin in nasal polyposis. *Allergy Asthma Immunol Res.* 2011;3:186–193.
- [32] Takai T, Chen X, Xie Y, Vu AT, Le TA, Kinoshita H, et al. TSLP expression induced via Toll-like receptor pathways in human keratinocytes. *Methods Enzymol.* 2014;535:371–387.
- [33] Han H, Xu W, Headley MB, Jessup HK, Lee KS, Omori M, et al. Thymic stromal lymphopoietin (TSLP)-mediated dermal inflammation aggravates experimental asthma. *Mucosal Immunol.* 2012;5:342–351.
- [34] Spergel JM. Atopic march: link to upper airways. *Curr Opin Allergy Clin Immunol.* 2005;5:17–21.
- [35] Cianferoni A, Spergel J. The importance of TSLP in allergic disease and its role as a potential therapeutic target. *Expert Rev Clin Immunol.* 2014;10:1463–1474.
- [36] Kemény L, Szabó K. Toll-like receptors link atopic march to the hygiene hypothesis. *J Invest Dermatol.* 2013;133:874–878.
- [37] Hussain I, Jain VV, Kitagaki K, Businga TR, O'Shaughnessy P, Kline JN. Modulation of murine allergic rhinosinusitis by CpG oligodeoxynucleotides. *Laryngoscope.* 2002;112:1819–1826.
- [38] Li M, Hener P, Zhang Z, Kato S, Metzger D, Chambon P. Topical vitamin D3 and low-calcemic analogs induce thymic stromal lymphopoietin in mouse keratinocytes and trigger an atopic dermatitis. *Proc Natl Acad Sci USA.* 2006;103:11736–11741.
- [39] Kim HJ, Kim YJ, Lee SH, Yu J, Jeong SK, Hong SJ. Effects of *Lactobacillus rhamnosus* on allergic march model by suppressing Th2, Th17, and TSLP responses via CD4(+)CD25(+)Foxp3(+) Tregs. *Clin Immunol.* 2014;153:178–186.
- [40] Weaver CT, Hatton RD. Interplay between the TH17 and TReg cell lineages: a (co-)evolutionary perspective. *Nat Rev Immunol.* 2009;9:883–889.
- [41] Akdis CA, Akdis M. Mechanisms of immune tolerance to allergens: role of IL-10 and Tregs. *J Clin Invest.* 2014;124:4678–4680.
- [42] Chauhan A, Singh M, Agarwal A, Paul N. Correlation of TSLP, IL-33, and CD4 + CD25 + FOXP3 + T regulatory (Treg) in pediatric asthma. *J Asthma.* 2015;52:868–872.
- [43] Lee JH, Yu HH, Wang LC, Yang YH, Lin YT, Chiang BL. The levels of CD4+CD25+ regulatory T cells in paediatric patients with allergic rhinitis and bronchial asthma. *Clin Exp Immunol.* 2007;148:53–63.
- [44] Grindebacke H, Wing K, Andersson AC, Suri-Payer E, Rak S, Rudin A. Defective suppression of Th2 cytokines by CD4CD25 regulatory T cells in birch allergics during birch pollen season. *Clin Exp Allergy.* 2004;34:1364–1372.
- [45] Kim do H, Sohn JH, Park HJ, Lee JH, Park JW, Choi JM. CpG oligodeoxynucleotide inhibits cockroach-induced asthma via induction of IFN- γ ⁺ Th1 Cells or Foxp3⁺ regulatory T cells in the lung. *Allergy Asthma Immunol Res.* 2016;8:264–275.
- [46] Tang J, Zhou X, Liu J, Meng Q, Han Y, Wang Z, et al. IL-25 promotes the function of CD4+CD25+ T regulatory cells and prolongs skin-graft survival in murine models. *Int Immunopharmacol.* 2015;28:931–937.
- [47] Yao X, Sun Y, Wang W, Sun Y. Interleukin (IL)-25: pleiotropic roles in asthma. *Respirology.* 2016;21:638–647.
- [48] Gordon ED, Locksley RM, Fahy JV. Cross-talk between epithelial cells and type 2 immune signaling. The role of IL-25. *Am J Respir Crit Care Med.* 2016;193:935–936.
- [49] Tworek D, Smith SG, Salter BM, Baatjes AJ, Scime T, Watson R, et al. IL-25 receptor expression on airway dendritic cells after allergen challenge in subjects with asthma. *Am J Respir Crit Care Med.* 2016;193:957–964.
- [50] Kleinjan A, Willart M, van Rijt LS, Braunstahl GJ, Leman K, Jung S, et al. An essential role for dendritic cells in human and experimental allergic rhinitis. *J Allergy Clin Immunol.* 2006;118:1117–1125.

- [51] Schroder NW, Maurer M. The role of innate immunity in asthma: leads and lessons from mouse models. *Allergy*. 2007;62:579–590.
- [52] Beeh KM, Kanniss F, Wagner F, Schilder C, Naudts I, Hammann-Haenni A, et al. The novel TLR-9 agonist QbG10 shows clinical efficacy in persistent allergic asthma. *J Allergy Clin Immunol*. 2013;131:866–874.
- [53] Casale TB, Cole J, Beck E, Vogelmeier CF, Willers J, Lassen C, et al. CYT003, a TLR9 agonist, in persistent allergic asthma - a randomized placebo-controlled Phase 2b study. *Allergy*. 2015;70:1160–1168.