

ORIGINAL ARTICLE

CpG-ODNs and Budesonide Act Synergistically to Improve Allergic Responses in Combined Allergic Rhinitis and Asthma Syndrome Induced by Chronic Exposure to Ovalbumin by Modulating the TSLP-DC-OX40L Axis

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Abstract— The experimental model of combined allergic rhinitis and asthma syndrome (CARAS) has shown that CpG oligodeoxynucleotides (CpG-ODNs) are potential inhibitors of type 2 helper cell-driven inflammatory responses. Currently available CpG-ODNs modestly inhibit allergic responses in CARAS, while a combination strategy for upper airway treatment by co-administration of CpG-ODNs and glucocorticoids may show good efficacy. This study aimed to assess the therapeutic effects of CpG-ODNs combined with budesonide (BUD) on upper and lower-airway inflammation and remodeling in mice with CARAS induced by chronic exposure to ovalbumin (OVA), exploring the possible underlying molecular mechanisms. A BALB/c mouse model of chronic CARAS was established by systemic sensitization and repeated challenge with OVA. Treatment with CpG-ODNs or BUD by intranasal administration was started 1 h after OVA challenge. Then, nasal mucosa and lung tissues were fixed and stained for pathologic analysis. The resulting immunologic variables and TSLP-DC-OX40L axis parameters were evaluated. Both CpG-ODNs and BUD intranasal administration are effective on reducing Th2-type airway inflammation and tissue remodeling. Co-administration of CpG-ODNs and BUD was more effective than each monotherapy in attenuating upper and lower-airway inflammation as well as airway remodeling in chronic

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Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10753-018-0779-6>) contains supplementary material, which is available to authorized users.

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CARAS. Notably, combination of CpG-ODNs with BUD modulated the TSLP-DC-OX40L axis, as demonstrated by decreased TSLP production in the nose and lung, alongside decreased TSLPR and OX40L in DC. Intranasal co-administration of CpG-ODNs and BUD synergistically alleviates airway inflammation and tissue remodeling in experimental chronic CARAS, through shared cellular pathways, as a potent antagonist of the TSLP-DC-OX40L axis.

KEY WORDS: Bronchial asthma; Allergic rhinitis; CpG oligodeoxynucleotides; Dendritic cells; Thymic stromal lymphopoietin.

INTRODUCTION

Allergic rhinitis (AR) and asthma coexist in a large proportion of patients. Indeed, 30–80% asthmatic patients suffer from AR. Meanwhile, almost 40% of patients with AR display asthmatic symptoms, suggesting a close relationship between the two ailments [1]. Therefore, combined AR and asthma syndrome (CARAS) was coined to describe concurrent rhinitis and asthma symptoms in clinical patients [2]. Mounting evidence suggests that AR is a risk factor for and often precedes asthma, while AR treatment might improve asthma symptoms [3–7]. Current guidelines for AR and asthma emphasize anti-inflammatory therapy. Glucocorticoids remain the first-line agents in asthma treatment [5, 8]. Although asthma is mostly well controlled by conventional therapies such as inhaled corticosteroids, approximately 10% of patients have uncontrolled asthma, which has been described as “severe asthma,” or “difficult-to-control asthma” [9–11], with a number of chronic asthmatic patients developing irreversible pulmonary function alterations. Severe asthma has a distinct pathophysiology including airway remodeling that contributes to the decreased effectiveness of ICS therapy. However, when high doses are required to control symptoms, adverse effects such as growth stunting in children [12], suppression of the hypothalamic-pituitary-adrenal (HPA) axis [13], and osteoporosis [14] may be observed. Therefore, alternatives or add-on drugs are required to solve this problem. Progress in the development of novel treatment strategies for asthma has been relatively slow, and current guidelines are primarily based on clinical symptoms rather than the underlying pathogenetic mechanisms. Immunomodulatory attempts have aimed at redirecting the associated inappropriate Th2 response toward allergens and inducing a counterbalancing Th1 or regulatory T cell response [15]. The central components in such immunotherapeutic attempts are TLRs, which are key players in the innate immune system that mediate recognition and response to various microbial and viral

pathogens [16]. CpG oligodeoxynucleotides (CpG-ODNs) are synthetic single-stranded DNAs containing unmethylated CpG motifs with immunostimulatory properties due to their resemblance to bacterial genomes. CpG-ODNs are recognized by TLR9 in certain innate immune cells such as dendritic cells (DCs) [17] and induce T helper (Th)1-type reactions [18] while concomitantly inhibiting preexisting Th2 responses; these activities are predicted to moderate Th2-dominated allergic states. We previously reported that intranasal administration of CpG-ODNs alone could transiently attenuate CARAS, possibly involving pulmonary thymic stromal lymphopoietin (TSLP) suppression [19, 20]. We also provided evidence that TSLP plays a pivotal role in the initiation and persistence of airway inflammation and remodeling in the context of chronic allergic asthma [21, 22]. In Th2 immune responses, TSLP plays a key role *via* induction of OX40-ligand (OX40L) on DCs, which in turn triggers the Th2 cell-mediated allergic cascade. Thus, the TSLP-DC-OX40L axis might be the principal pathway in inflammatory cascades associated with atopic diseases [23].

Although our previous findings demonstrated that CpG-ODNs may have therapeutic benefits in acute CARAS [19, 20], whether CpG-ODNs can inhibit the signs of airway remodeling associated with chronic CARAS has not been addressed. Currently, therapy with inhaled corticosteroids, although disputes still exist, may be one of the important strategies in preventing airway remodeling [24, 25]. However, it remains unclear whether intranasal administration of CpG-ODNs could be combined with corticosteroids for the control of CARAS. In this study, local administration of CpG-ODNs and/or budesonide (BUD) was adopted in mice with chronic CARAS to assess the effects of CpG-ODNs on upper and lower-airway inflammation and remodeling, as well as the synergistic effects of CpG-ODNs and glucocorticoids. We also explore the underlying mechanism, with focus on the TSLP-DC-OX40L axis, which is considered to be critical in Th2-dominated allergic diseases.

MATERIALS AND METHODS

Experimental Animals

Female BALB/c mice from Laboratory Animal Center of Southern Medical University, China (SCXK 2016-0041; 8–22 g; 6–8 weeks) were maintained under specific pathogen-free conditions under a 12 h/12 h light-dark cycle with food and water *ad libitum*.

Antigen Sensitization, Challenge, and Treatment

Experimental animals were randomly divided into five groups ($n = 12/\text{group}$), including control (sensitized and challenged with PBS), CARAS (sensitized and challenged with OVA), CpG-ODNs, BUD, and combination (CpG-ODNs + BUD) groups. An ovalbumin (OVA)-induced chronic CARAS asthma model was established as previously described by our team [19, 20], with slight modifications. To induce CARAS, BALB/c mice were first sensitized on days 0, 7, and 14 by intraperitoneal injection of 20 μg OVA (Sigma, USA) emulsified in 2 mg $\text{AL}(\text{OH})_3$ (YuanMu, Shanghai, China) in a total volume of 200 μL phosphate-buffered saline (PBS). For non-sensitized control mice, only PBS was injected. One week later, the animals were challenged intranasally in the awake state by instilling a 10- μL droplet of OVA (2 mg/mL) or PBS into each nostril using a micropipette. Next, the animals were challenged intranasally for three successive days a week for 3 cycles. Four days after the last intranasal (i.n.) OVA or PBS was administered, in the awake state, mice were challenged with 5 ml of 2% OVA (CARAS group) or 5 ml PBS using the aerosol exposure technique for 30 min on three consecutive days per week for up to 6 weeks. The mice were administered a nasal instillation of all drugs in a volume of 10–20 μL into the bilateral cavities and kept in the supine position to ease inhalation for a few minutes. In the CpG-ODN group, 1 h after challenge, the mice were intranasally administered CpG-ODNs (20 μg per nostril in 10 μL of 0.9% NaCl). Synthetic CpG-ODNs with two CpG motifs (5'-TCCATGACGTTCTGACGTT-3') (Sangon Biotechnology, Shanghai, China) were used as reported previously [19]. The BUD group received intranasal administration of BUD (10 μg per nostril in 20 μL Budesonide Inhalation Suspension; *pulmicort respules*® AstraZeneca, UK) 1 h after OVA administration. The dosages of CpG-ODNs and BUD were obtained from a pilot study [19, 20]

and another report [26]. An outline of the experimental protocol is presented in Fig. 1.

Assessment of Frequencies of Nose Scratching and Sneezing

To examine the effects of CpG-ODNs or BUD on nasal symptomatology, we performed behavior observation of mice by counting the number of nose-sneezing and rubbing movements for 10 min immediately after the final nasal challenge as previously reported [27–29]. Sneezing was characterized by an explosive expiration just after deep inspiration. Scratching was characterized by an external peri-nasal scratch with the animal's forelimbs [30].

Cell Differentiation and Cytokine Quantification in the Bronchoalveolar Lavage Fluid

Bronchoalveolar lavage fluid (BALF) samples were obtained 24 h after the final OVA exposure as described previously [20] and centrifuged at 2000 rpm for 10 min at 4 °C; the resulting supernatants were stored at –80 °C for further analysis. Then, TSLP, TGF- β 1, IL-4, IL-5, IL-13, and IFN- γ levels in the BALF were quantified with specific ELISA kits (TSLP kit from YuanMu, Shanghai, China; IL-4 kit from LDEBIO, Guangzhou, China; IL-5, IL-13, and IFN- γ kits from MultiSciences, Hangzhou, China) according to the manufacturers' instructions. Cells pelleted from BALF samples were used to assess inflammation *via* eosinophils cell count as described in our previous reports [19–21].

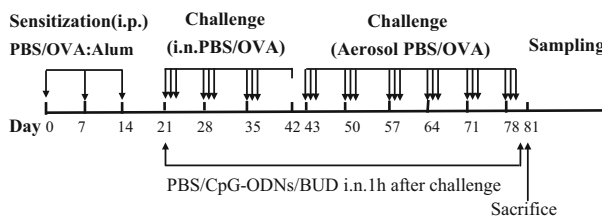


Fig. 1. Schematic representation of treatment protocols. Mice were sensitized to ovalbumin (OVA) by three intraperitoneal (i.p.) injections on days 0, 7, and 14 with OVA (20 μg) absorbed to alum (2 mg) and challenged intranasally (i.n.) starting on day 21 with OVA (10 μL , 2 mg/ml) thrice weekly for 3 weeks. This was followed by OVA aerosol challenge three times a week for six consecutive weeks. Therapeutic treatment with PBS, CpG-ODNs, and BUD (i.n.) was started 1 h after the nasal or aerosol challenge. The mice were sacrificed on day 81 by cervical dislocation, and bronchoalveolar lavage fluid (BALF) samples were collected; bone marrow-derived DCs were generated, and nose and lung specimens were obtained for histological analysis.

Histological Examination

Mouse heads were fixed with 10% formalin, and nasal tissues were separated from head muscles and skin. The right lung lobe from each animal was removed for histological examination. To assess upper- and lower-airway inflammation, nasal and lung tissue sections were stained with hematoxylin and eosin (H&E) for the detection of inflammatory infiltrates. The severity of peribronchial inflammation and eosinophil count in the submucosal region of the nasal septum were scored by a blinded expert according to previously described features [20]. To assess airway remodeling, lung sections were stained with periodic acid Schiff stain (PAS) for goblet cell and mucus production assessment, or with Masson's trichrome for collagen level determination, as described previously by our team [21].

Immunohistochemistry

The expression levels of TGF- β 1 and α -smooth muscle actin (α -SMA) in lung tissues were assessed by immunohistochemical staining. Samples were incubated with antibodies specific to TGF- β 1 (Bioss, Beijing, China) and α -SMA (Abcam, UK) overnight at 4 °C. Staining was detected with biotinylated secondary antibodies (goat anti-rabbit horseradish peroxidase-IgG secondary antibodies, Abcam, UK) and the DAB reagent (Allway Biotechnology, Guangzhou, China) at 37 °C for 30 min. Semi-quantitative analysis for the intensity of positive staining of TGF- β 1 and α -SMA in lung tissue sections were measured through Image-Pro plus 6.0 software (Media Cybernetics, USA) as described previously [31, 32]. All the images were taken using the same microscope and camera sets. The intensity of positive staining in tissue sections was analyzed by average optic density, which means integrated optical density (IOD) per stained area under each examined field (IOD/area) [33].

Immunofluorescence

Nasal specimens were fixed, paraffin embedded, and sectioned into 5- μ m-thick slices. After blocking with non-immune serum, the specimens were subsequently incubated with primary and secondary antibodies, respectively. Anti-TSLP (Novus, Millipore, USA) were used as primary antibodies, and secondary antibodies were rabbit anti-goat IgG (Southern biotech, USA). Counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI, Sigma, USA). Immunofluorescent images were obtained under a fluorescence microscope and analyzed by IOD/area

mentioned above using the Image-Pro Plus software (version 6, Media Cybernetics, USA).

Western Blot

TSLP protein amounts in lower-airway tissues were further assessed by Western blotting as previously reported by our team [20]. Briefly, total protein in supernatant samples obtained from left lung tissue lysates were quantified with a BCA Protein Assay Kit (KeyGEN Biotech, Nanjing, China), according to the manufacturer's instructions. Equal amounts of total protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto immobilon-P transfer membranes (Merck Millipore, USA). After overnight incubation with primary antibodies against TSLP (Novus, USA) or GAPDH (KangChen Biotech, Shanghai, China; used as the internal reference control) at 4 °C, the membranes were treated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (Southern biotech, USA) for 1 h. The signals were assessed with the Image-Pro Plus software (version 6, Media Cybernetics, USA).

Real-Time Quantitative PCR

The mRNA levels of TSLP and Th1/Th2 cytokines in lung samples were measured by real-time PCR (ABI PRISM® 7500 Sequence Detection System, USA). Total RNA in left lung samples was isolated with TRIzol (Invitrogen), as described previously by our team [20]. PCR was performed using the following specific primers: TSLP (forward, 5'-CCTTCCTCAGCACACAATTG-3'; Reverse, 5'-TGATAAGCTGGCTCACAGAA-3'), IL-4 (forward, 5'-CAGCAAGCACTCAACGGAAT-3'; reverse, 5'-CGTCCTCTGAACGACCAACA-3'), IL-5 (forward, 5'-GCTGCTGATGTGGAAAGATT-3'; reverse, 5'-AGCTGTCCACTGTTGGTAAAG-3'), IL-13 (forward, 5'-ACCCAACAACCACTATGCT-3'; reverse, 5'-TGCACTCATTGGTGGAGGTA-3'), IFN- γ (forward, 5'-CCTTCCTCAGCACACAATTG-3'; Reverse, 5'-TGATAAGCTGGCTCACAGAA-3'), and GAPDH (forward, 5'-GGCCTCCAAGGAGTAAGAAA-3'; reverse, 5'-GCCCTCCTGTTATTATGG-3'). GAPDH was used for normalization, and relative gene expression levels were derived by the $2^{-\Delta\Delta C_t}$ method.

Generation of Bone Marrow-Derived DCs

Bone marrow-derived DCs (BMDCs) were generated as described previously [34, 35], with slight modifications. Briefly, bone marrow cells from femur and tibia specimens

were flushed out with 5 ml of PBS using a syringe. Red cell depletion was performed with the ACK lysis buffer, and the remaining cells were cultured in RPMI-1640 (Hyclone, USA) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, USA), 4 mM L-glutamine, 25 mM HEPES, 50 μ M 2-mercaptoethanol, 100 U/ml penicillin (Hyclone, USA), and 100 μ g/ml streptomycin (Hyclone, USA). Cells (1×10^6 /well) were seeded in six-well flat-bottom plates containing recombinant murine GM-CSF (500 U/ml) and IL-4 (1000 U/ml) (PeproTech, USA) and incubated at 37 °C in a humidified environment containing 5% CO₂. Every other day, half of the culture medium was replaced with fresh medium containing GM-CSF and IL-4. On day 8, non-adherent cells were harvested and resuspended in serum-free RPMI-1640. Cytokine concentrations in cell culture supernatants were measured with specific ELISA kits (IL-12p70, LDEBIO, Guangzhou, China). IL-12 mRNA levels in DCs were analyzed by real-time quantitative PCR, with the following primers: IL-12 (forward, 5'-CCATCGCTTCTCTCATATTC-3'; reverse, 5'-CTTCTGCTAACACATTGAGG-3'), GAPDH (forward, 5'-GGCCTCCAAGGAGTAAGAAA-3'; reverse, 5'-GCCCCTCCTGTTATTATGG-3').

Mixed Lymphocyte Reaction

This assay evaluates the stimulatory ability of DCs to alter T cell cytokine production. DCs from different groups were used as stimulator cells. Allogeneic T-lymphocytes were obtained from BALB/c mice using the lymphocyte separation medium (Ficoll PaquePLUS, GE). The purified lymphocytes were cultured in complete RPMI 1640 medium supplemented with 10% FCS in 96-well plates at 37 °C for 48 h. The stimulated DCs and T cells were co-cultured in 96-well flat-bottom tissue culture plates at a ratio of 1:10 with serum-free RPMI-1640 as described previously [35]. Cells were allowed to proliferate at 37 °C in 5% CO₂ for 72 h. The collected culture supernatants were used for cytokine level assessment with specific ELISA kits (IL-4: LDEBIO, Guangzhou, China; IL-5, IL-13, and IFN- γ : MultiSciences, Hangzhou, China).

Flow Cytometry

Single-cell suspensions of DCs were stained for CD40 (eBioscience), CD80 (eBioscience), CD86 (eBioscience), OX40L (eBioscience), TSLPR (R&D), and IL-7R (R&D). Events were gated according to their FSC and SSC properties, with dead cells excluded from the final analysis. Data were acquired on a BD FACS Aria

flow cytometer (BD Biosciences) and analyzed using CellQuest Pro (BD Biosciences) [21].

Measurement of OVA-Specific IgE

A total of 0.7 ml blood was collected from each mouse by cardiac exsanguination. OVA-specific IgE levels were determined by ELISA, as described previously [19, 20]. Results were expressed in pg/ml.

Statistical Analysis

For statistical evaluations, one-way analysis of variance (ANOVA) was used to calculate the overall *P* value, with Bonferroni's multiple comparison tests. The statistical software package STATA 10.0 (Stata Corp LP) was used for all analyses. Results are mean $\pm$ standard deviation (SD). *P* < 0.05 was considered statistically significant.

RESULTS

Intranasal CpG-ODNs and BUD Synergistically Inhibit TSLP in Upper and Lower Airways of Mice with Chronic OVA Exposure-Associated CARAS

TSLP is a necessary and sufficient factor in the initiation of allergic airway inflammation [21]; therefore, TSLP expression levels were assessed in nasal mucosa and lung tissues to determine whether TSLP production is reduced in the upper and lower airways of mice with CARAS after treatment with CpG-ODNs and BUD. First, TSLP expression was evaluated in the lower airway. Quantitative RT-PCR, ELISA, and Western blotting showed that TSLP mRNA levels in lung tissues and protein amounts in both BALF and lung tissue samples were increased in mice with CARAS, compared with control values (all *P* < 0.01, Fig. 2a–c), corroborating our previous observations [20]. Treatment with either CpG-ODNs or BUD resulted in reduced TSLP mRNA and protein levels, compared with values obtained for untreated mice with CARAS (all *P* < 0.01, Fig. 2a–c), with BUD appearing to be more effective than CpG-ODNs. Meanwhile, mice simultaneously treated with CpG-ODNs and BUD showed dramatically decreased TSLP mRNA levels compared with the monotherapy groups (both *P* < 0.01, Fig. 2a). Next, TSLP expression was evaluated in nasal specimens by immunofluorescence and immunohistochemistry. As shown in Fig. 2f, g, TSLP was expressed in mice with CARAS, with lower levels in control mice. Intranasal administration of either CpG-ODNs or BUD in mice with CARAS resulted in decreased

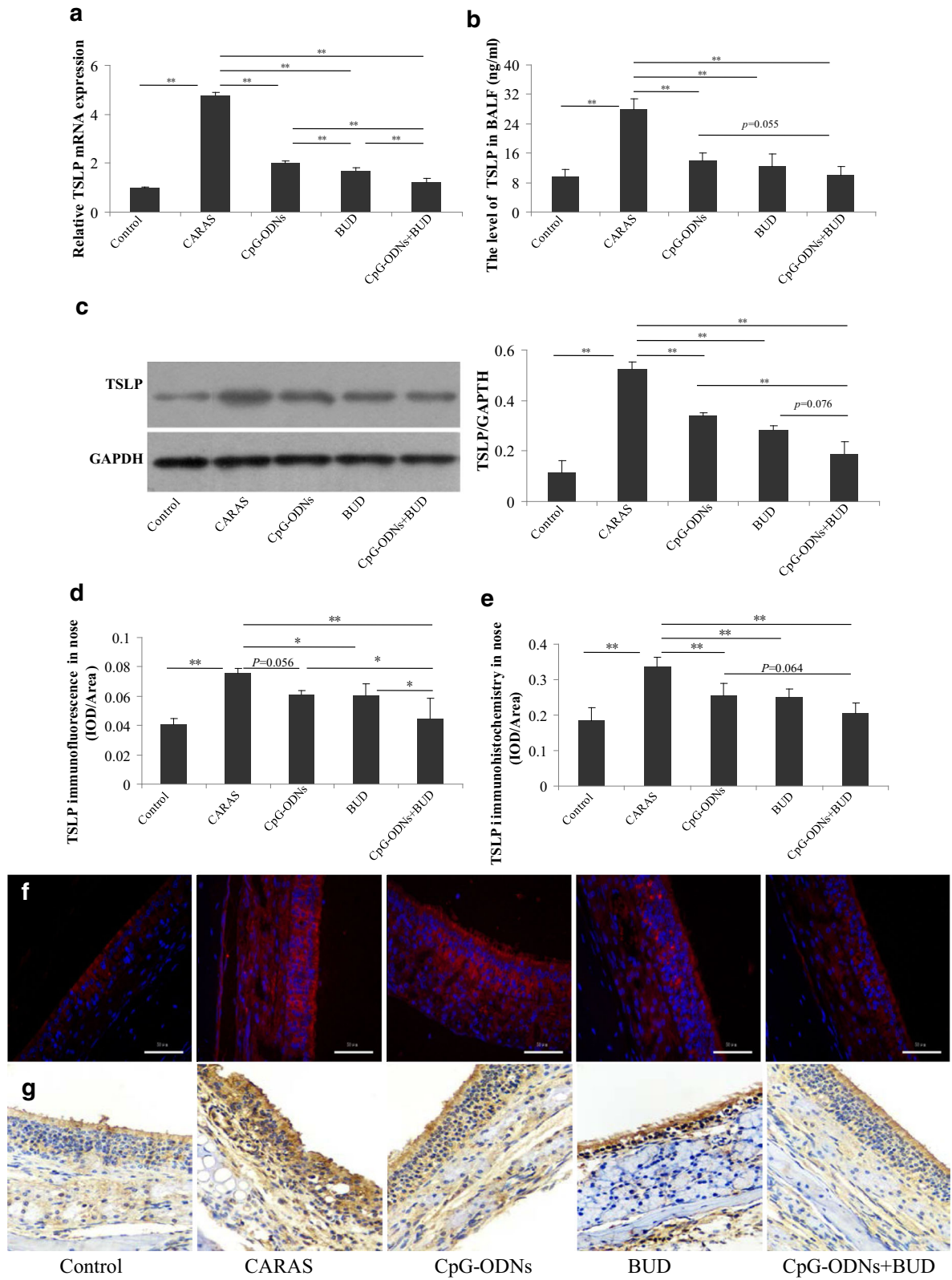


Fig. 2. Co-administration of CpG-ODNs and BUD inhibits TSLP expression in the upper and lower airways of CARAS mice induced by chronic exposure to ovalbumin. **a** Relative *Tslp* mRNA levels in lung samples were detected by real-time PCR. **b** TSLP protein concentrations in BALF samples were detected by ELISA. **c** Western blot for detecting TSLP protein expression in lung tissues. GAPDH was used as an internal control. TSLP protein expression was also analyzed by immunofluorescence (**d**) and immunohistochemistry (**e**). **f** Representative immunofluorescent photomicrographs with TSLP staining (red), with DAPI-stained nuclei appearing blue in paraffin-embedded nasal tissues in the indicated mouse groups. Original magnification, $\times 400$; scale bars, 50 μm . **g** Representative immunohistochemical photomicrographs with TSLP staining in nasal samples. Data are mean $\pm$ SD ($n = 5$ or 6 animals per group). * $P < 0.05$; ** $P < 0.01$, compared with the counterpart groups.

nasal TSLP expression. Notably, intranasal administration of combined CpG-ODNs and BUD in mice with CARAS further reduced nasal TSLP levels (Fig. 2d, e). These data indicated that prolonged OVA exposure resulted in TSLP overproduction in both upper and lower airways of animals with CARAS, and intranasal administration of CpG-ODNs and BUD combination might synergistically reduce TSLP expression in upper and lower airways.

Intranasal Administration of Combined CpG-ODNs and BUD Inhibits Cellular Inflammation in the Nasal Mucosa and Lower Airway to a Greater Degree Compared with Single Treatments, Potently Relieving Nasal Symptoms

As TSLP downregulation in asthmatic mice could lead to significantly less airway eosinophils [36], we next compared nasal and lung histology among the different groups, to further evaluate the effects of CpG-ODNs and/or BUD on upper and lower-airway inflammation. Compared with PBS-treated control mice, all treatment groups showed significantly reduced eosinophil counts in the BALF, with more obvious changes in the CpG-ODNs + BUD combination group (Supplementary Fig. 1A). CARAS mouse models showed thickened bronchial mucosa, lumen stenosis, high rates of inflammatory cell (eosinophil and lymphocyte) infiltration (Supplementary Fig. 1E), which were rarely detected in sham-sensitized mice. Meanwhile, CpG-ODNs and BUD synergistically alleviated airway inflammation, and only mild peribronchial infiltrates (Supplementary Fig. 1E) and lower lung inflammation (Supplementary Fig. 1B) were observed. Consistent with lung findings, mice with CARAS developed significant nasal submucosa eosinophilia compared with control

animals (Supplementary Fig. 1F). In addition, fewer eosinophils in nasal tissues were observed in the CpG-ODNs and BUD combination group (Supplementary Fig. 1F). Furthermore, mice with CARAS exhibited goblet cell hyperplasia in the nasal mucosa, which was inhibited by treatment with CpG-ODNs and BUD, in a synergistic manner (Supplementary Fig. 1G). To examine the effects of CpG-ODNs and/or BUD on nasal symptomatology, nasal rubbing and sneezing events were counted for 10 min immediately after the last nasal challenge. Expectedly, sensitized mice developed increased nasal scratching and sneezing after exposure to OVA, while markedly less nasal rubbing and sneezing movements were observed in mice with CARAS after intranasal CpG-ODNs or BUD treatment, in comparison with those that received no therapy (both $P < 0.01$, Supplementary Figure 1C and D). Meanwhile, intranasal administration of combined CpG-ODNs and BUD potently relieved nasal symptoms compared with both monotherapies (all $P < 0.01$, Supplementary Fig. 1C and D). Collectively, intranasal therapy with CpG-ODNs and BUD in mice with CARAS substantially decreased the severity of allergic airway responses, in a synergistic manner.

Intranasal Treatment with CpG-ODNs Inhibits Airway Structural Remodeling

Inhibition of TSLP not only eliminates the established airway inflammation but also prevents airway structural remodeling [21, 22]. We, therefore, evaluated the effects of intranasal CpG-ODNs and BUD on airway remodeling. Airway wall thickening is associated with peribronchial collagen deposition, and sub-epithelial fibrosis is a characteristic feature of airway remodeling [21]. Peribronchial collagen deposition was assessed by Masson's trichrome staining. As shown in Fig. 3a, repeated exposure to OVA for six consecutive weeks resulted in increased airway wall thickness, with enhanced peribronchial collagen deposition in OVA-exposed mice compared with the PBS-treated controls. Furthermore, whether this strong phenotype could be suppressed by therapeutic interventions was assessed. Intranasal administration of CpG-ODNs or BUD during OVA exposure resulted in attenuated peribronchial collagen deposition (Fig. 3a), reducing airway wall thickness, compared with the PBS-treated controls (Fig. 3a). Goblet cell hyperplasia and metaplasia are common features of chronic asthmatic airways [37]. Next, we evaluated airway goblet cell and mucus amounts by PAS staining. Continuous respiratory exposure to OVA caused significant mucus hypersecretion in the lumen as well as goblet cell hyperplasia in the airway epithelium compared

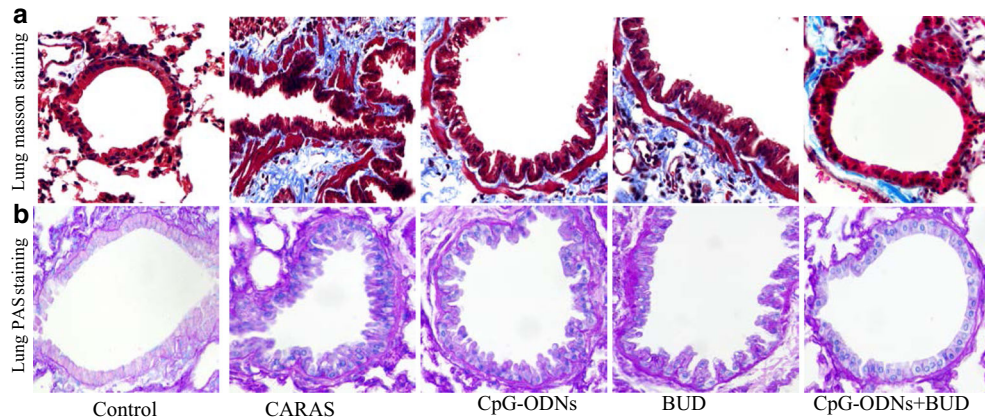


Fig. 3. Intranasal CpG-ODNs and BUD therapy inhibits airway structural remodeling. Representative photomicrographs of Masson's trichrome- (a) and PAS (b)-stained lung sections from the indicated mouse groups. Original magnification, $\times 400$.

with PBS-treated control mice (Fig. 3b). Pretreatment with CpG-ODNs resulted in decreased mucus hypersecretion and goblet cell hyperplasia associated with prolonged OVA exposure, compared with PBS-treated mice (Fig. 3b). As shown previously, α -SMA expression acts as a marker of fibroblast differentiation into myofibroblasts, which are responsible for fibrotic collagen matrix deposition [38]. TGF- β 1, with important immunomodulatory and fibrogenic characteristics, has been implicated in inflammation and remodeling processes [24]. We found that intranasal administration of CpG-ODNs and BUD resulted in reduced TGF- β 1 levels in the BALF (confirmed by ELISA, Fig. 4a) and lung tissues (immunohistochemistry in Fig. 4b, d) and decreased α -SMA expression in lung tissues (immunohistochemistry in Fig. 4c, e). However, as monotherapy with either CpG-ODNs or BUD obviously reduced TGF- β 1 and α -SMA expression levels almost to control levels, the combination therapy was not likely to have increased effects on TGF- β 1 and α -SMA expression levels compared with the monotherapies. These findings demonstrated that intranasal treatment of mice with CARAS with CpG-ODNs and BUD prevented structural remodeling of the airways by inhibiting peribronchial collagen deposition and goblet cell upregulation, effects accompanied by decreased TGF- β 1 and α -SMA levels.

Combination Therapy with CpG-ODNs and BUD Have Additive Effects on Th1/Th2 Homeostasis Modulation in the Lung, BALF, and Splenocytes

As TSLP conditions the immune system to induce a robust Th2 response, initiating chronic airway inflammation and structural remodeling in mice with chronic asthma [21], to assess the contribution of CpG-ODNs/BUD

treatment to Th1/Th2 cytokine homeostasis in the CARAS model, we analyzed the levels of Th1/Th2-associated cytokines in lung tissue and BALF specimens from experimental animals. As expected, the mRNA levels of Th2-type cytokines (IL-4, IL-5, and IL-13) were higher in lung samples from mice with CARAS compared with control animals (all $P < 0.01$, Fig. 5a–c). Intranasal treatment with combined CpG-ODNs and BUD resulted in reduced lung mRNA levels of IL-4, IL-5, and IL-13 compared with the untreated mice with CARAS or the CpG-ODNs or BUD monotherapy groups (all $P < 0.01$, Fig. 5a–c), with the exception of IL-13 mRNA, whose levels were not more pronouncedly reduced by CpG-ODNs and BUD combination compared with BUD monotherapy ($p = 0.069$, Fig. 5c). Furthermore, untreated mice with CARAS exhibited significantly lower mRNA levels of the lung Th1-type cytokine IFN- γ , compared with control mice ($P < 0.01$, Fig. 5d). However, this change was reversed to some extent by intranasal administration of combined CpG-ODNs and BUD ($P < 0.01$, Fig. 5d). In agreement, combination therapy with CpG-ODNs and BUD resulted in further reduced protein levels of Th2-type cytokines in the BALF, showing a statistically significant decrease compared with CpG-ODNs ($P < 0.01$, Fig. 5f, g) but not BUD ($P > 0.05$, Fig. 5f, g) monotherapy. However, as monotherapy with either CpG-ODNs or BUD resulted in obviously decreased Th2-type cytokines in the BALF, the combination therapy might not show more effects on Th2-type cytokines in the BALF compared with the monotherapies. Interestingly, combined treatment resulted in remarkably enhanced mRNA levels of the Th1-type cytokine IFN- γ , which was not observed at the protein level (Fig. 5h). Consistently, significantly higher levels of OVA-induced Th2-type

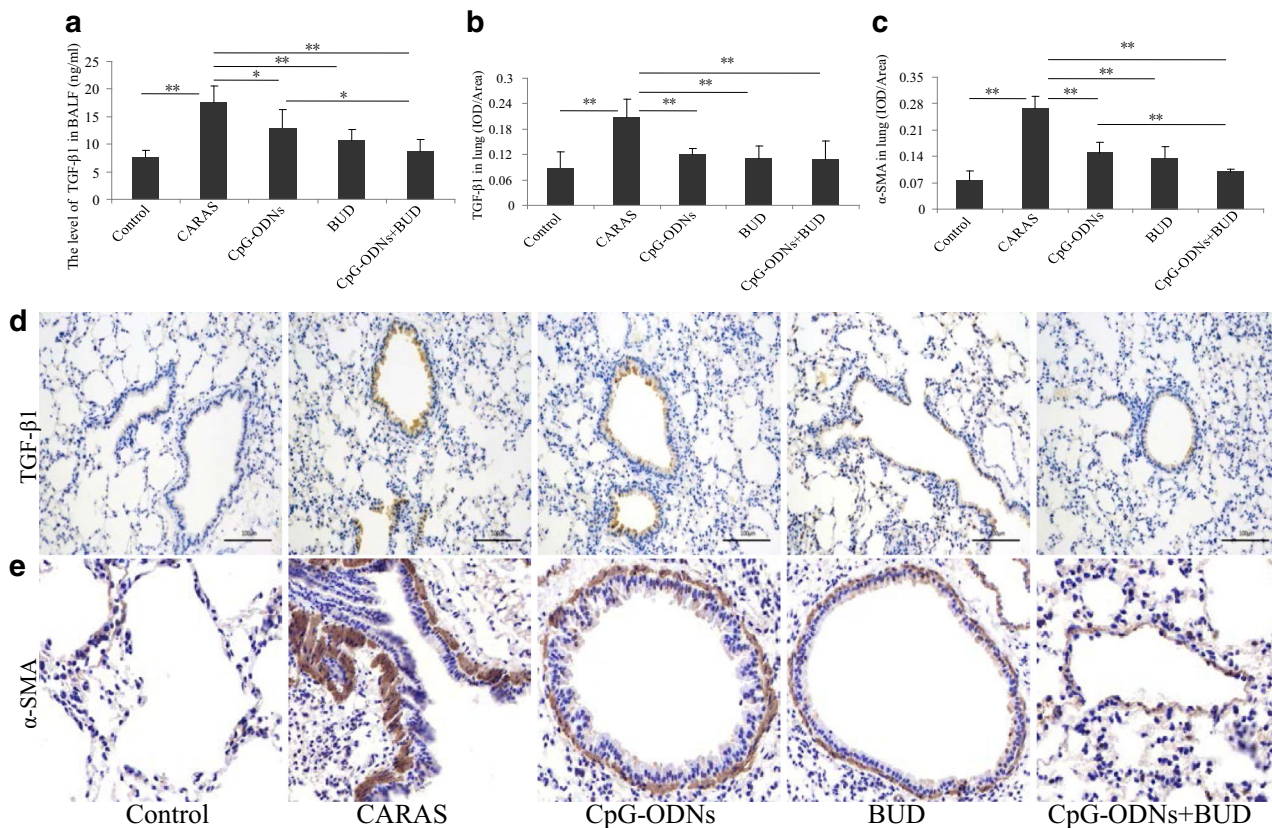


Fig. 4. Intranasal administration of CpG-ODNs and BUD reduces TGF-β1 and α-SMA levels. The levels of TGF-β1 were determined by ELISA in BALF samples (a) and by immunohistochemistry in lung tissue specimens (b). The levels of α-SMA were determined by immunohistochemistry in lung tissue samples (c). Representative photomicrographs of immunohistochemical staining of lung sections for TGF-β1 (d) and anti-α-SMA (e) detection. Original magnification, × 200.

cytokines (IL-4 and IL-5) were observed in spleen cells from mice with CARAS compared with control animals (both $P < 0.05$, data not shown). Splenocytes and lymphocytes in BALB/c mice after intranasal CpG-ODNs and BUD showed significantly lower levels of secreted IL-4 and IL-5, to a greater degree than non-treated animals or those administered single treatments (all $P < 0.05$, data not shown). These findings clearly indicated that a robust Th2 response was induced in mice with CARAS, with intranasal CpG-ODNs and BUD treatments synergistically suppressing the dominant production of Th2-associated cytokines while enhancing the levels of Th1-associated cytokines, thereby modulating Th1/Th2 homeostasis in the CARAS model. Thus, the CpG-ODNs and BUD combination may offer a therapeutic application as a potent Th1-type inducer and Th2-type inhibitor. In agreement with the above results, serum levels of OVA-specific IgE were elevated in the CARAS mouse model, and both CpG-ODNs and BUD effectively reduced IgE amounts in this

model (all $P < 0.01$, data not shown), with no significant differences after single treatment use. Compared with CpG-ODNs monotherapy, administration of combined CpG-ODNs and BUD further inhibited OVA-specific IgE levels ($P < 0.05$, data not shown).

Intranasal CpG-ODNs and BUD Synergistically Inhibit the Expression Levels of DC Surface Markers and Affect the Potential of DCs to Polarize Lymphocytes

As TSLP is a potent activator of DCs [39], which play a vital role in sensitization to inhaled antigens and asthma development [40], we therefore assessed whether DCs might be involved in TSLP inhibition by CpG-ODNs and BUD. First, cell-surface receptors of DCs, required for T cell activation [41], were assessed. As shown in Fig. 6, intranasal treatment with combined CpG-ODNs and BUD of mice with CARAS resulted in reduced CD40, CD80, and CD86 expression by DCs, in a synergistic manner,

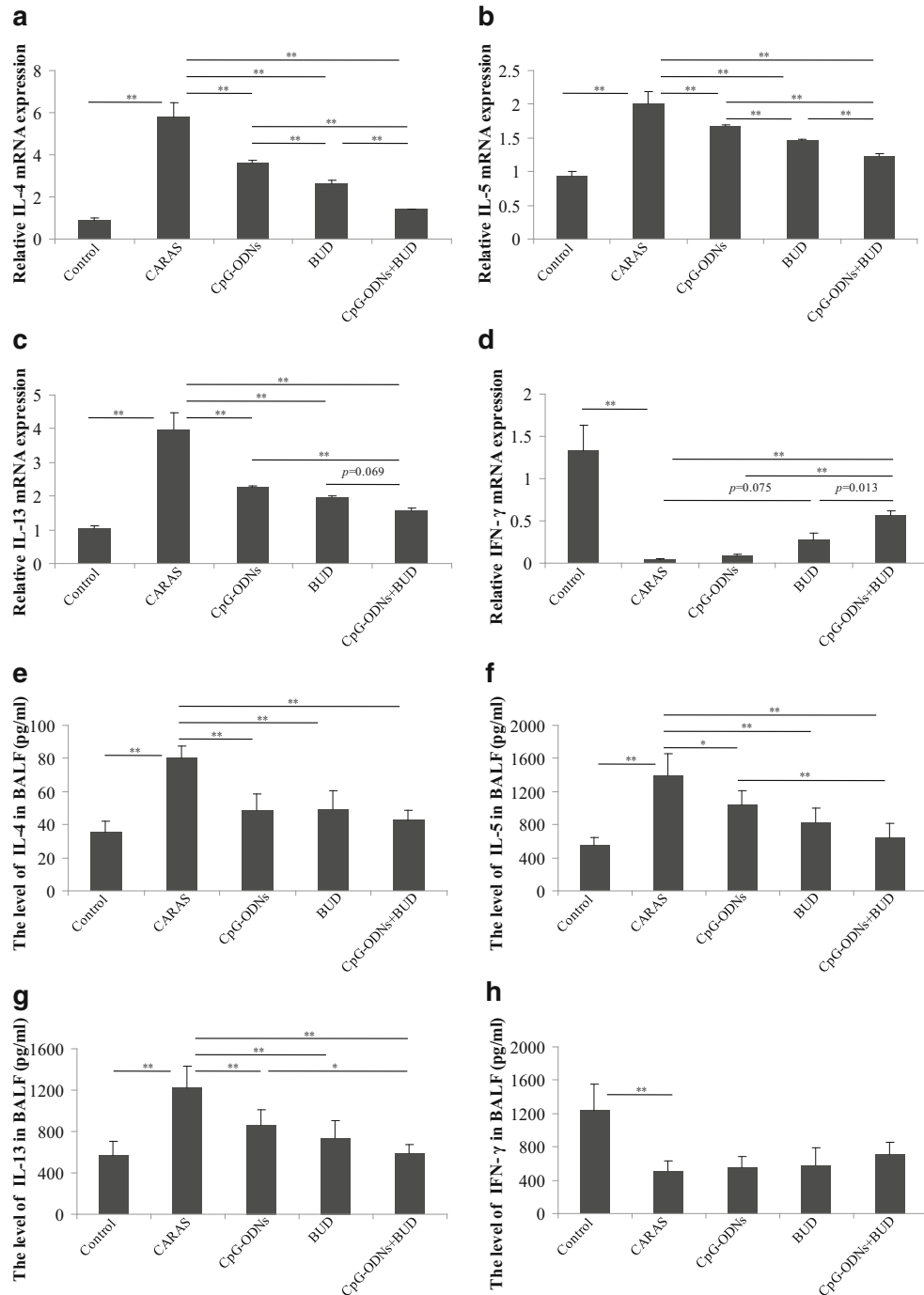


Fig. 5. Intranasal CpG-ODNs and BUD therapy synergistically alters Th1/Th2 type responses in the lower airway. **a–d** Relative mRNA expression levels of Th1/Th2 cytokines in lung tissues from mice, as assessed by real-time PCR. **e–h** ELISA for the detection of Th1/Th2-associated cytokines in the BALF. **i** Serum OVA-specific IgE amounts, as measured by ELISA. Data are mean $\pm$ SD from six independent experiments. * $P < 0.05$; ** $P < 0.01$ compared with the counterpart groups.

compared with control mice with CARAS, suggesting that maturation and activation of lung DCs were substantially affected by CpG-ODNs and BUD. Strikingly, decreased TSLP receptor (TSLPR) and IL-7R α levels in DCs after intranasal treatment with CpG-ODNs and BUD of mice with CARAS were observed, indicating that TSLPR inhibition also affected DC function. The TSLP receptor is a heterodimer that consists of the common γ -like receptor chain called TSLP receptor (TSLPR) and IL-7 receptor α chain (IL-7R α). As DCs express OX40L, a Th2 polarizing molecule [42], we further examined the ability of combined CpG-ODNs and BUD to activate DCs producing OX40L. The increased OX40L levels in DCs were dramatically inhibited by pretreatment with combined CpG-ODNs and BUD, compared with non-treated mice with CARAS or those administered single treatments (Fig. 6). Additionally, given that TSLP induces the expression of maturation markers on DCs and increases their ability to polarize lymphocytes into the Th2 phenotype [43], we assessed the capability of DCs in different groups to stimulate and polarize T cell response by co-culturing them with T cells, an assay used to evaluate the stimulatory ability of DCs to alter T cell proliferation and cytokine production [34]. Consistently, significantly higher levels of OVA-induced Th2-type cytokines (IL-4, IL-5, and IL-13) and lower levels of the Th1-type cytokine IFN- γ were observed in DC-T cell supernatants from mice with CARAS compared with those from control animals (all $P < 0.01$, Fig. 7a–d). DC-T cell supernatants in the CpG-ODNs or BUD groups showed significantly lower levels of IL-4, IL-5, and IL-13 compared with control animals with CARAS (all $P < 0.05$, Fig. 7a–c). Furthermore, intranasal administration of combined CpG-ODNs and BUD inhibited the significant potential toward Th2 polarization, as evidenced by reduced levels of IL-5 and IL-13, alongside elevated IFN- γ levels in DC-T cell supernatants, compared with single treatments (both $P < 0.05$, Fig. 7b–d). Moreover, because the profile of secreted cytokines in DCs can affect T cell differentiation [44], with IL-12p70 playing a key role in Th1 polarization of CD4⁺ T cells [35], we next evaluated IL-12p70 production by DCs isolated from asthmatic mice. As shown in Fig. 7e, f, intranasal treatment with CpG-ODNs or BUD significantly increased IL-12p70 amounts, both at the protein and mRNA levels, compared with control animals with CARAS (all $P < 0.05$). In addition, intranasal administration of combined CpG-ODNs and BUD further increased IL-12p70 protein and mRNA amounts, compared with the two monotherapies (all $P < 0.05$). These findings demonstrated that intranasal CpG-ODNs and BUD synergistically inhibited the

expression levels of DC surface markers, decreased OX40L in DCs, and affected the potential of DCs to polarize lymphocytes.

DISCUSSION

Asthma and AR are major public health problems because of their frequencies and effects on the quality of life, school performance, and economy [45]. Strong anatomical, epidemiologic, pathophysiologic, clinical, and therapeutic evidences in upper and lower airways support the connection between the so-called united airway disease (UAD) or CARAS [1]. Asthma and AR are both manifestations of a shared inflammatory process and require an integrated diagnostic and therapeutic approach for global disease control [1]. Wert et al. reported a decrease in asthma symptoms and AR after intranasal corticosteroid treatment for AR, and a recent meta-analysis confirmed the beneficial effects of intranasal steroids in AR [46]. The interaction between the nose and lung in allergic airways disease is a bidirectional process; indeed, treatment of AR might improve asthma symptoms [5, 6]. This is the theoretical basis of the present study assessing whether reducing upper airway inflammation by targeting CpG-ODNs to the upper airway might improve lower-airway outcomes of chronic CARAS. Currently, treatment with corticosteroids is considered one of the most important strategies for the treatment of allergic diseases such as AR and asthma. However, difficult-to-control asthma has a distinct pathophysiology including airway remodeling that contributes to the decreased effectiveness of inhaled corticosteroid therapy. Alternatives or add-on drugs such as CpG-ODNs could be potential additive effects with administration of inhaled corticosteroids for the control of allergic diseases. Therefore, in the present study, we selected BUD as positive control and assessed whether CpG-ODNs have synergistic effects with corticosteroids.

Asthma is a multi-step immune disorder characterized by mass infiltration of various inflammatory cells, including eosinophils, mast cells, and Th2 cells; DCs, however, play even more important roles as upstream inducers of the allergic inflammatory cascade, not only through their powerful abilities of processing and presenting inhaled antigens but also *via* their unique properties of orchestrating immune responses [36]. Therefore, DCs constitute the key to understanding the pathogenesis of and devise efficient treatments for allergic diseases. Tissue-derived factors, such as TSLP, contribute to the specific airway microenvironment directing DCs toward a pro-allergic or anti-

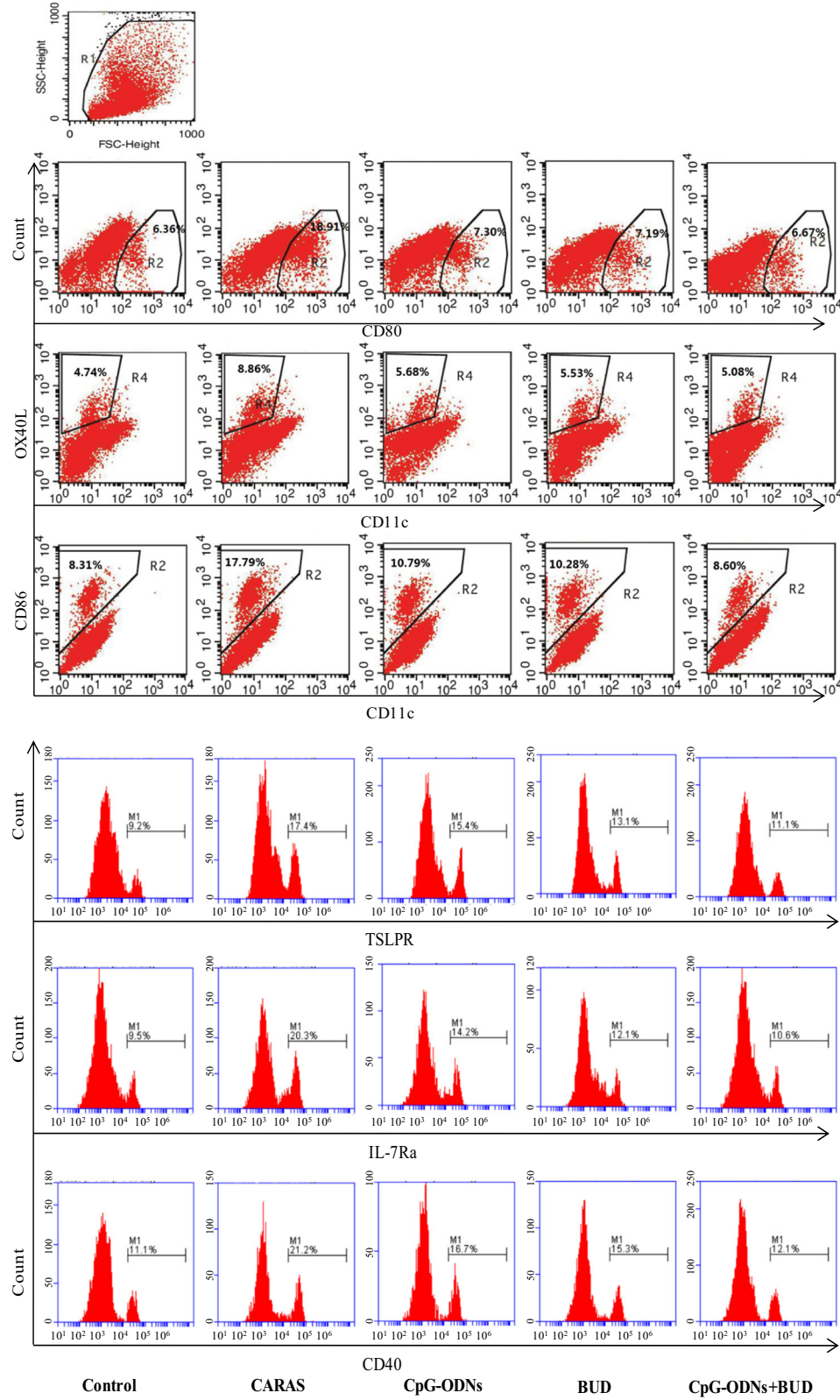


Fig. 6. CpG-ODNs and BUD intranasally administered to mice with CARAS synergistically inhibit the cell-surface phenotype in bone marrow-derived dendritic cells. DCs were generated from bone marrow samples from different groups BALB/c mice. Non-adherent cells collected at day 8 were assessed for CD40, CD80, CD86, OX40L, TSLPR, and IL-7Ra expression by flow cytometry.

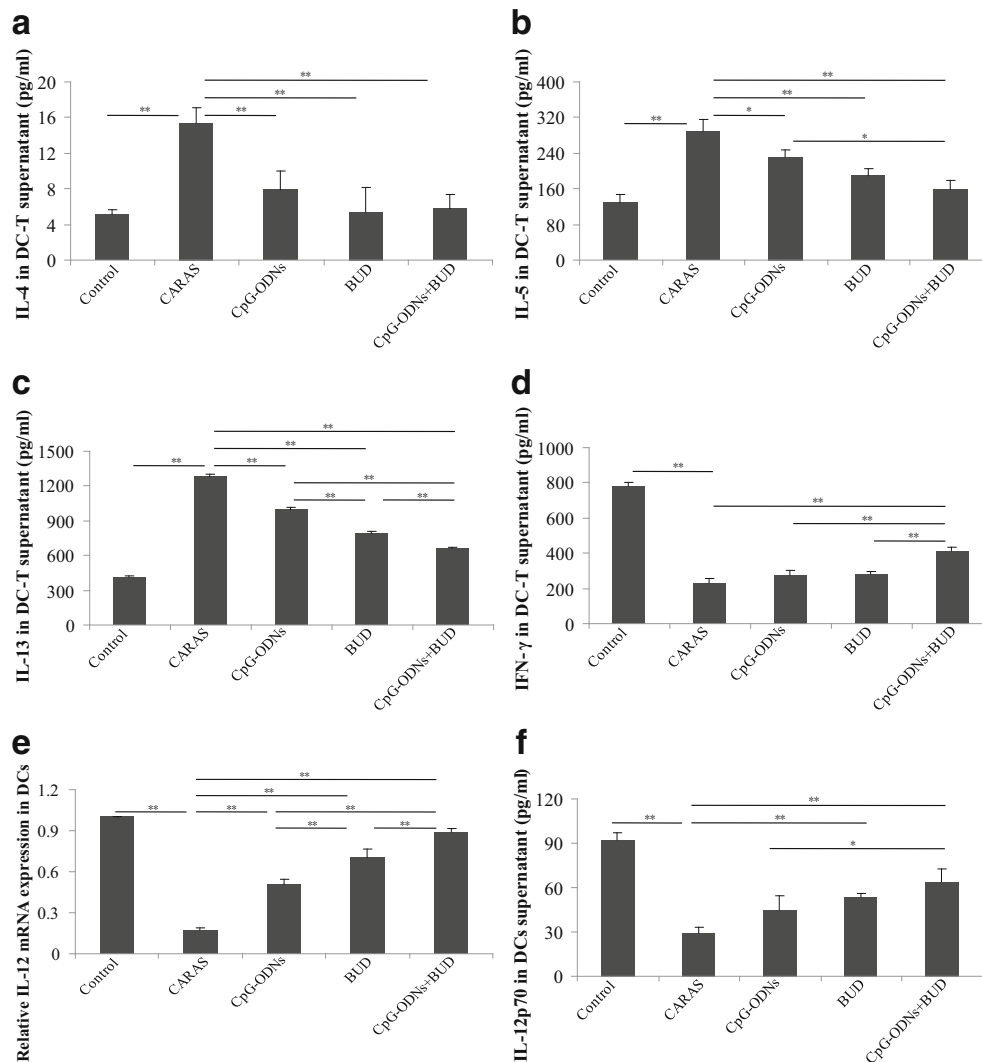


Fig. 7. CpG-ODNs and BUD administered intranasally synergistically affect the potential of DCs to polarize lymphocytes. **a-d** Th2-type cytokine production levels in DC-T culture supernatants were determined by ELISA. **e, f** IL-12p70 (mRNA and protein) amounts in DCs isolated from various mouse groups.

allergic function [47, 48]. Meanwhile, OX40L is a TSLP-induced surface marker on DCs that mediate inflammatory Th2 cell differentiation [21]. TSLP-activated DCs upregulate OX40L, which then interacts with OX40 on T cells, resulting in the polarization of naive T cells toward the Th2 pathway [21].

This study confirmed that prolonged exposure to OVA in CARAS mice resulted in TSLP overproduction in the upper and lower airways (Fig. 2), an effect associated with elevated levels of Th2 cytokines (Fig. 5) and increased eosinophil infiltration (Supplementary Fig. 1), suggesting that TSLP production is responsible for the

extension of Th2-biased inflammation, and TSLP production in the upper and lower airways upon OVA exposure might be the early event in the immune cascade leading to subsequent allergic inflammation. Given that chronic OVA exposure contributed to TSLP overproduction, we assessed whether TSLP affects DC activation, by flow cytometry. As expected, chronic OVA exposure resulted in increased DC maturation, with higher expression levels of the DC surface molecules OX40L, CD40, CD80, and CD86, compared with control values (Fig. 6). We previously demonstrated that TSLP is overproduced in the chronic house dust mite-induced asthma model [21], as well as in the

acute CARAS mouse model [20]. This finding was extended by the present study, which revealed that high TSLP expression in the lower airway of chronic CARAS mice was associated with increased OX40L expression on DCs (Fig. 6), alongside OVA-induced chronic CARAS airway remodeling, characterized by sub-epithelial fibrosis and peribronchial collagen deposition (Figs. 3 and 4). Taken together, these data underscored the importance of the TSLP-DC-OX40L axis in Th2-mediated inflammation and raised the feasibility of developing TSLP signaling-oriented therapies for CARAS.

Consistent with previous findings from acute CARAS models [20], AR treatment with intranasal CpG-ODNs improved lower-airway inflammation in chronic CARAS mice (Supplementary Fig. 1). It should be emphasized that although intranasal administration of CpG-ODNs was effective in shifting the immune response from Th2 to Th1, the Th1/Th2 cytokine ratio remained shifted toward the Th2 pathway, compared with PBS-treatment controls (Fig. 5). This finding suggested that inhibition of allergic responses by CpG-ODNs alone was modest. Interestingly, such effect on cytokine production was significantly enhanced after co-administration of BUD (Supplementary Fig. 1 and Fig. 5). Using OVA-driven chronic CARAS mouse models, this study showed that co-administration of CpG-ODNs and BUD showed pronounced efficacy in abrogating the cardinal features of asthma, with significantly reversed nose and lung pathologies, attenuated IgE production, reduced lower-airway eosinophilic inflammation, reversed splenocyte and BALF Th2-type responses, and alleviated nasal symptoms, suggesting that the combined use of CpG-ODNs and BUD may have synergistic effects in this chronic CARAS animal model.

Evidences suggest that infiltrating inflammatory cells, such as eosinophils, play an active role in airway remodeling by promoting the activation of fibroblasts and endothelial cells [49]. Eosinophils are among the major sources of fibrotic factors, which induce the differentiation of airway submucosal fibroblasts into myofibroblasts that synthesize and secrete collagen into the airway submucosal space, causing airway fibrosis [49]. In addition, a shift in Th1/Th2 homeostasis to Th2 responses also causes airway wall structural remodeling [21]. Consistent with the above findings, mice with CARAS pretreated with CpG-ODNs and BUD failed to develop the salient features of airway remodeling, including peribronchial collagen deposition, goblet cell hyperplasia, and mucus overproduction (Fig. 3). Airway fibrosis was confirmed by TGF- β 1 and α -smooth muscle actin expression in lungs, as CpG-ODNs and BUD controlled TGF- β 1 levels and α -ASM mass in

OVA-induced chronic CARAS (Fig. 4). These findings suggest that CpG-ODNs administered through the intranasal route not only eliminates established airway inflammation but also plays a protective role in airway remodeling.

Our data partially differ from findings by other groups. For instance, Duechs MJ et al. demonstrated that although the TLR9 agonist CpG-ODNs reduce allergen-specific IgE, eosinophil, collagen I, and IL-4 and IL-5 amounts, while increasing IFN- γ amounts and strongly enhancing IL-10 levels in the lung of severe triple allergen asthma models, no effects on airway hyper-reactivity and airway smooth muscle mass were found [50]. The discrepancy in airway remodeling is most likely attributable to different animal models used in the two studies (steroid-insensitive triple allergen [OVA, cockroach, and house dust mite] model *versus* steroid-sensitive OVA model).

To further explore the mechanism underlying the anti-inflammatory effects of intranasal instillation of CpG-ODNs and BUD, we focused on TSLP-DC-OX40L axis, the critical players in asthmatic responses, DCs preferentially expressing costimulatory molecules, IL-12, and TSLPR [36]. TSLPR is a novel type 1 cytokine receptor, which, combined with IL-7 receptor- α (IL-7R α), constitutes the TSLP receptor complex [51]. Costimulatory molecules on antigen-presenting cells such as DCs play a critical role in determining the type of T cell response. OX40L is an important costimulatory molecule expressed on a wide variety of cells, including DCs. Interactions between OX40 and its ligand are involved in many inflammatory diseases, including allergies. Increased OX40L expression was shown to favor Th2 polarization of T cells by antigen-presenting cells [52]. A critical DC cytokine involved in Th polarization is interleukin (IL)-12, which is a major factor in Th1 differentiation [53]. Accordingly, low IL-12 levels in activated DCs are associated with the risk of developing Th2 immune responses to allergens, whereas high amounts seem to provide protection from allergic diseases [53]. Firstly, we assessed TSLP expression upon co-administration of CpG-ODNs and BUD in both upper and lower airways and found that intranasal administration of CpG-ODNs and BUD was pronouncedly efficient in regulating TSLP levels in upper and lower airways, as reflected by decreased TSLP protein and mRNA expression levels in the nose, BALF, and lung tissues (Fig. 2). This confirmed our previous observation in the acute CARAS model, that TSLP levels are decreased in CARAS mice treated with CpG-ODNs [20]. To further explore the role of TSLP in DC-mediated Th2 priming, we evaluated the effects of CpG-ODNs and BUD on the capability of DCs to stimulate and polarize T cells. Interestingly,

pretreatment with CpG-ODNs resulted in lower OX40L, CD40, CD80, CD86, and IL-12 amounts, as well as reduced TSLPR and IL-7R α expression on DCs (Figs. 6 and 7) while promoting Th2 differentiation by triggering the production of pro-allergic cytokines (IL-4, IL-5, and IL-13) (Fig. 5). CpG-ODNs and BUD showed synergistically pronounced effects in regulating the above biomarkers, suggesting that administration of CpG-ODNs and BUD appeared to remodel the DC-initiated TH1/2 response by downregulating Th2 cytokines while upregulating Th1 cytokines, in part, to modulate DC maturation and TSLPR. Since maturation of airway DCs is a prerequisite in priming allergic inflammation [36], reducing these activities by TSLPR blockade with CpG-ODNs and BUD might be responsible for the suppressed Th2 priming and inflammatory response observed in this study. These findings corroborate the cytokine profile observed in BALF samples from CpG-ODNs and BUD-treated mice with chronic CARAS (Fig. 5), stressing the importance of TSLPR-mediated signaling in DC conditioning toward pro-allergic functions (Figs. 6 and 7). Meanwhile, blocking TSLP with CpG-ODNs and BUD in total splenocyte culture resulted in boosted production of IL-4, alongside mildly reduced IFN- γ production (data not shown), indicating that IL-4 is involved in the mechanism underlying the effects of TSLPR inhibition in Th2-mediated allergic inflammation. As shown above, increased TSLP expression in the nasal mucosa and lung tissues, along with increased TSLPR and OX40L levels in DCs, was significantly alleviated in mice with CARAS after treatment with CpG-ODNs and BUD, suggesting that modulation of the TSLP-DC-OX40L axis accounted, at least in part, for the reduced severity of chronic CARAS in mice. However, only limited conclusions can be drawn from the current results. Further studies are required to confirm these findings and to precisely define relevant pathways and other effects of TSLP on airway structural cells using *ex vivo* experiments and *in vivo* chronic OVA-exposed models.

In conclusion, prolonged OVA exposure in CARAS mice causes eosinophil influx, increasing the levels of Th2 cytokines and peribronchial collagen deposition in the airways, effects accompanied by increased TSLP levels in the upper and lower airways. Meanwhile, intranasal administration of combined CpG-ODNs and BUD effectively inhibits airway eosinophil infiltration, providing a significant preventive benefit with the potential to inhibit airway remodeling. The alleviating effects of CpG-ODNs and BUD on chronic CARAS mice were achieved by inhibition of TSLP of upper and lower airway, along with its abilities of endowing DCs express OX40L and

promoting Th2 differentiation by triggering the production of pro-allergic cytokines (IL-4, IL-5, and IL-13). These data could, to some extent, explain why intranasal co-administration of CpG-ODNs and BUD in chronic CARAS mice resulted in reduced TSLP activation of DCs, which in turn downregulates OX40L, *via* the OX40L-OX40 pathway, priming naive T cells to differentiate into Th2 cells. Based on this and previous reports, CpG-ODNs are extremely important contributors to Th1/Th2 cytokine homeostasis as well as airway structural remodeling and might provide effective synergism to corticosteroids for the management of AR and asthma. Therefore, CpG-ODNs may be further assessed for the clinical treatment of AR and asthma.

ACKNOWLEDGEMENTS

We gratefully thank Qing Liu for helping us to establish the animal model. We also thank Rong Yao for administrative assistance. This manuscript was edited for English language by MedSci.

Funding This study was supported by the National Natural Science Foundation of China (grant number 81470220, 81470219), Science and Technology Planning Project of Guangdong Province, China (grant number 2016A020215220), Science and Technology Program of Guangzhou, China (grant number 201707010076), and Natural Science Foundation of Guangdong Province, China (grant number S2013010015990). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

All the animal experiments conformed to the principles for the care and use of animals in biomedical research. The study protocol was approved by the ethics committee of animal experiments of the Vaccine Research Institute of Sun Yat-Sen University vivarium ([2014]2-11).

Competing Interest. The authors declare that they have no competing interests.

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Supplementary Fig.1.

