

Hypothesis & Experience
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Suppressive effect of environmental tobacco smoke on murine Th2 cell-mediated nasal eosinophilic inflammation

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Conflict of Interest

The authors have no financial conflicts of interest.

Author Contributions

Conceptualization: Osamu Kaminuma, Tomoe Nishimura. Data curation: Osamu Kaminuma, Tomoe Nishimura. Formal analysis: Osamu Kaminuma, Tomoe Nishimura. Funding acquisition: Osamu Kaminuma. Methodology: Osamu Kaminuma, Mayumi Saeki, Noriko Kitamura. Project administration: Osamu

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ABSTRACT

Background: Environmental tobacco smoke (ETS) exposure is recognized as a risk factor for the development of various respiratory diseases.

Objective: In this study, the effect of ETS on allergen-immunized and allergen-specific Th2 cell-transferred murine eosinophilic inflammation models and that of cigarette smoke extract (CSE) and nicotine on allergen-induced Th2 cell proliferation and interleukin (IL)-4 production were investigated.

Methods: Ovalbumin (OVA)-immunized and OVA-specific Th2 cell-transferred BALB/c mice were exposed to ETS and were challenged with OVA. Then, the number of inflammatory cells in the nasal mucosa and nasal hyperresponsiveness (NHR) were assessed. The effects of CSE and nicotine on the allergen-induced proliferative response of and IL-4 production by Th2 cells were determined *in vitro*.

Results: In OVA-immunized and Th2 cell-transferred mice, allergen-induced NHR and nasal eosinophil infiltration were significantly suppressed by ETS exposure, whereas the accumulation of neutrophils was rather enhanced. Allergen-specific Th2 cell proliferation and IL-4 production were inhibited by coculture with CSE. The same effects were induced by nicotine, though the effect on proliferation was relatively weak.

Conclusion: Regardless of its harmful effect, ETS suppresses NHR, probably through the inhibition of Th2 cell responses.

Keywords: Environmental tobacco smoke; Mouse; Nasal hyperresponsiveness; Nicotine; Th2 response

INTRODUCTION

Environmental tobacco smoke (ETS) has been recognized as a risk factor for the development of respiratory diseases such as lung cancer, allergic asthma, and chronic obstruction pulmonary disease (COPD) [1]. Moreover, ETS exposure is strongly associated with

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respiratory symptoms in adults [2-4] and children [5, 6]. In animal models, ETS exposure has been shown to enhance allergen sensitization, bronchial hyperresponsiveness (BHR), and eosinophilia [7, 8]. However, several clinical studies failed to find an association between ETS exposure levels and asthma severity [9, 10]. In some murine models of asthma, ETS exposure inhibits airway mucus formation, allergen-specific antibody production, and BHR [11, 12]. Therefore, the exact effect of ETS on respiratory diseases remains unclear.

Allergic rhinitis (AR) is characterized by several nasal symptoms, including sneezing, rhinorrhea, and nasal congestion, following provocation with the specific allergen. AR patients are characterized by the submucosal accumulation of inflammatory cells, including eosinophils, neutrophils, and T cells, accompanied by nasal hyperresponsiveness (NHR) [13]. We have recently demonstrated that allergen-specific CD4⁺ T cells play a crucial role in the development of NHR [14, 15].

Although the nasal mucosa is a primary target of ETS exposure, the effect of ETS on AR has been poorly investigated. Therefore, by employing the Th2 cell transfer model as well as the conventional allergen immunization model, we assessed the effect of ETS exposure on allergen-induced nasal eosinophilic inflammation, including NHR. To elucidate the mechanisms of ETS-mediated responses, the effects of cigarette smoke extract (CSE) and nicotine on allergen-induced Th2 cell responses were also examined *in vitro*.

MATERIALS AND METHODS

Animals

Six-week-old female BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). DO11.10/RAG-2^{-/-} mice were maintained for allergen-specific Th2 cell preparation as described previously [16]. The experimental protocols were approved by the Animal Use and Care Committee of Tokyo Metropolitan Institute of Medical Science.

Cigarette smoke extract

CSE was prepared by the method described by Carp and Janoff [17] with slight modifications. Briefly, 10-mL Dulbecco's modified eagle medium (DMEM) nutrient mixture F12-HAM culture medium bubbled with 150 mL of cigarette smoke from 4 filter-removed and ignited cigarettes (Peace, 21 mg tar and 1.9-mg nicotine per cigarette, Japan Tobacco Inc., Tokyo, Japan) was filtered through a 0.22- μ m filter (Merck, Darmstadt, Germany). This solution was considered 100% CSE and diluted to designated concentrations.

In vitro polarization of allergen-specific Th2 cells

Allergen-specific Th2 cells were prepared as described previously [16, 18]. Briefly, OVA-specific naïve CD4⁺ T cells were isolated from splenocytes of DO11.10/RAG-2^{-/-} mice by positive selection using CD4 microbeads and a magnetic-activated cell sorting system (Miltenyi, Bergisch Gladbach, Germany). Cells were cultured with X-ray-irradiated splenocytes in DMEM-nutrient mixture F12-HAM medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum. At the start of culture, 0.3- μ M synthetic OVA323-339 peptide (Scrum Inc., Tokyo, Japan), 10-U/mL recombinant IL-2 (Shionogi, Osaka, Japan), 10-U/mL recombinant IL-4 (PeproTech, Rocky Hill, NJ, USA), and 10- μ g/mL anti-IFN- γ monoclonal antibody (R4-6A2, eBioscience, San Diego, CA, USA) were added. Seven days after the stimulation, cells were harvested and used for the adoptive transfer and *in vitro* experiments.

Exposure to ETS

The exposure to ETS was performed according the previous report [19] with modifications. Mice were placed in a 9-L plastic chamber for 15 minutes, which was filled with ETS from 2 ignited cigarettes. This exposure was repeated twice a day with 10-minute intervals. Control mice were exposed to fresh room air according to the same schedule.

Allergen-induced nasal responses

In the allergen-immunized model, mice were sensitized by an intraperitoneal injection of 20 μg OVA (Sigma-Aldrich) emulsified with 2.25-mg alum (Thermo Fisher Scientific, Waltham, MA, USA) on days 0, 7, 14, and 21 of the experiment. Each day on days 35–39 and 42–46, mice were exposed to ETS, and 30 minutes later, they were challenged with an intranasal (i.n.) administration of 600- μg OVA dissolved in 20- μL saline or saline alone (Fig. 1A). In the Th2 cell transfer model, polarized Th2 cells (2×10^7) were intravenously injected in each BALB/c mouse on day 0, and these mice were exposed to ETS and challenged with OVA or saline each day on days 1–5 and 8–12 (Fig. 1B). In these models, NHR was assessed 6 hours after the last challenge by counting the number of sneezes for 5 minutes just after the administration of 10- μL histamine (100 mM; Nacalai tesque, Kyoto, Japan) [14]. Inflammatory cells in the nasal lavage fluid were classified by means of morphological criteria as described previously [20]. These models did not exhibit any inflammatory features in the lower airways [21].

Allergen-induced Th2 cell responses *in vitro*

Th2 cells (1×10^5) were incubated with X-ray-irradiated splenocytes (2×10^5) and 0.3- μM OVA peptide with or without CSE or nicotine (Nacalai tesque) for 72 hours. Th2 cell proliferation was assayed using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). The stimulation index was calculated as the ratio between stimulated and unstimulated wells as described previously [22]. The concentration of IL-4 in the culture supernatant was determined by enzyme-linked immunosorbent assay (ELISA) with Mouse IL-4 ELISA Ready-SET-GO (eBioscience) according to the manufacturer's protocol. The detection limit of this assay system was 31 pg/mL. The effects of CSE and nicotine

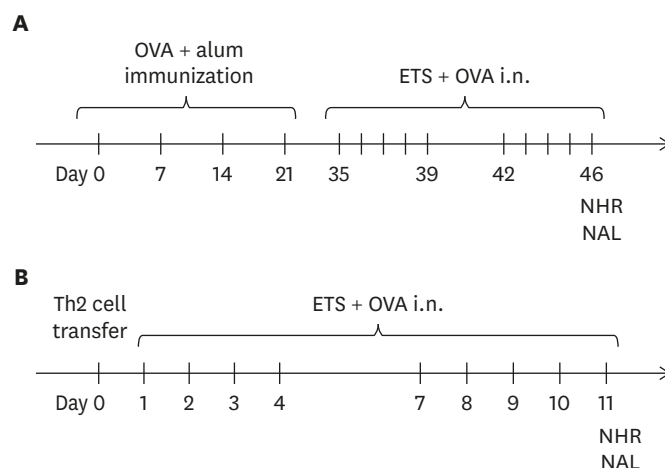


Fig. 1. Timeline of the experimental protocol. (A) In the allergen immunization model, mice were immunized 4 times with an intraperitoneal injection of ovalbumin (OVA) plus alum once a week. On days 35–39 and 42–46, mice were exposed to environmental tobacco smoke (ETS) and challenged with intranasal (i.n.) administration of OVA. (B) In the Th2 cell transfer model, after the transfer of polarized Th2 cells on day 0, mice were exposed to ETS and challenged with OVA on days 1–5 and 8–12. The nasal hyperresponsiveness (NHR) assessment and nasal lavage (NAL) were performed 6 hours after the last challenge.

are presented as percent stimulation index and IL-4 production compared with allergen-stimulated control.

Statistical analysis

The results are presented as the arithmetic mean \pm standard error of the mean. Statistical analysis was performed using 1-way analysis of variance and Dunnett multiple comparison test. $p < 0.05$ was considered to indicate statistical significance.

RESULTS

ETS suppressed allergen-induced nasal inflammation

To evaluate the effect of ETS on NHR, allergen-immunized mice were exposed to ETS and then challenged with OVA. Following the repeated allergen challenge, significant NHR was induced, as evidenced by the augmentation of the histamine-evoked sneezing response, compared with the saline-challenged control mice (Fig. 2A). At the same time, there was a significant induction of eosinophil and neutrophil infiltration into the nasal cavity (Fig. 2B). The allergen-induced NHR and eosinophil accumulation were significantly suppressed by ETS exposure (Fig. 2A, B), whereas the migration of neutrophils was further augmented (Fig. 2B). These parameters were not affected by ETS exposure alone.

Next, to examine the effect of ETS on Th2 cell-mediated nasal inflammation, allergen-specific Th2 cells were established from DO11.10/RAG-2^{-/-} splenocytes by *in vitro* stimulation culture. After confirming the adequate differentiation of the Th2 subset by evaluating cytokine production [18], Th2 cells were adoptively transferred to BALB/c mice. Similar to the results for the allergen immunization model, NHR (Fig. 3A) and nasal eosinophil migration (Fig. 3B) were significantly induced by OVA challenge, whereas obvious neutrophil accumulation was not seen (Fig. 3B). The allergen-induced NHR and eosinophil migration in Th2 cell-transferred mice were significantly suppressed by ETS exposure, though neutrophil migration was rather induced ($p = 0.067$) (Fig. 3B).

Effects of CSE and nicotine on Th2 cell responses

To investigate whether the suppression of allergen-induced nasal responses by ETS was caused by the down-regulation of Th2 cell-mediated responses, the effect of CSE on the proliferation of and IL-4 production by Th2 cells was investigated. OVA-specific Th2 cells

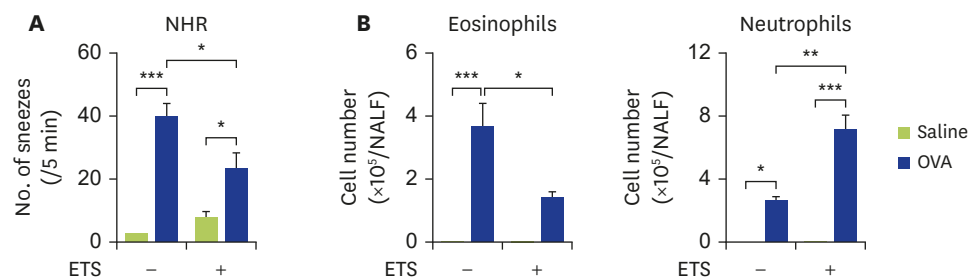


Fig. 2. Effect of environmental tobacco smoke (ETS) on allergen-induced nasal hyperresponsiveness (NHR) and cellular infiltration in the nasal cavity of allergen-immunized mice. Allergen-immunized mice were exposed to ETS and challenged with ovalbumin (OVA). (A) Six hours after the last challenge, NHR was evaluated by counting the number of sneezes evoked by histamine as described in the Materials and Methods. (B) Then, the number of eosinophils and neutrophils in the nasal lavage fluid (NALF) was determined. Data are expressed as the mean \pm standard error of the mean of 4–6 mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

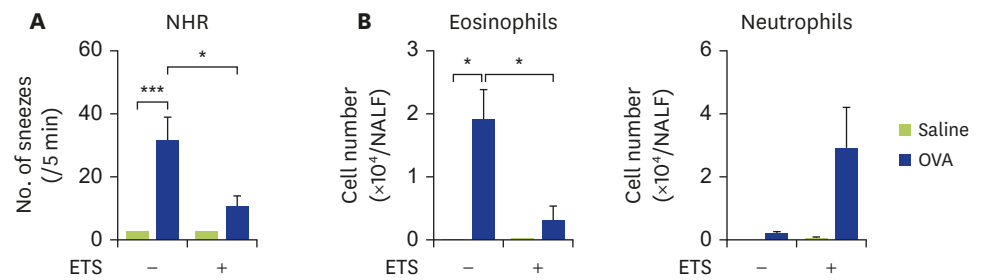


Fig. 3. Effect of environmental tobacco smoke (ETS) on allergen-induced nasal hyperresponsiveness (NHR) and cellular infiltration in the nasal cavity of Th2 cell-transferred mice. Th2 cell-transferred mice were exposed to ETS and challenged with ovalbumin (OVA). Six hours after the last challenge, NHR (A) and the number of cells in the nasal lavage fluid (NALF) (B) were assessed as described in Fig. 2. Data are expressed as the mean \pm standard error of the mean of 4–6 mice. * $p < 0.05$ and *** $p < 0.001$.

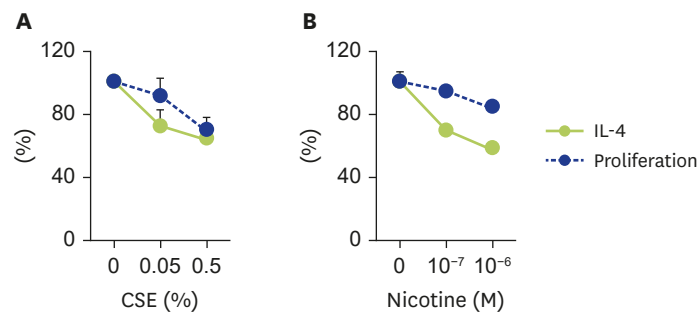


Fig. 4. Effects of cigarette smoke extract (CSE) and nicotine on the proliferative response of and interleukin (IL)-4 production by ovalbumin-specific Th2 cells. Polarized Th2 cells were stimulated with allergen in the presence or absence of CSE (A) and nicotine (B) for 72 hours. Then, the proliferative response and the concentration of IL-4 in the culture supernatant were assayed. Data are expressed as the mean \pm standard error of the mean of quadruplicate cultures of the percentages of stimulated (100%) and unstimulated (0%) control cells.

were cultured with X-ray irradiated splenocytes plus OVA peptide with or without CSE. Upon stimulation with OVA peptide, Th2 cells displayed an allergen-specific proliferative response (stimulation index = 8.04 ± 0.58). Furthermore, the concentration of IL-4 in the culture supernatant of Th2 cells was significantly elevated by OVA peptide stimulation (10.6 ± 0.3 ng/mL) compared with the unstimulated control (below the detection limit). The allergen-induced proliferative response of and IL-4 production by Th2 cells were similarly decreased by the addition of CSE in dose-dependent manners (Fig. 4A). These findings suggest that the down-regulation of Th2 cell responses was included in the ETS-mediated suppression of allergen-induced nasal responses.

To evaluate the mechanisms of the CSE-mediated suppression of Th2 cell activation, the effect of a representative CSE component, nicotine, was then examined. Similar to CSE, nicotine diminished the proliferation of and IL-4 production by Th2 cells in concentration-dependent manners, though the effect on proliferation was relatively weak (Fig. 4B). The inhibitory effect of CSE on Th2 cell responses seemed to be caused, at least in part, by the nicotine component of CSE.

DISCUSSION

Aside from the many investigations on its relationship with various respiratory diseases, the effect of ETS on the pathogenesis of AR, including NHR, has not been sufficiently examined.

In this study, the significant suppression of eosinophil infiltration into the nasal mucosa and NHR by ETS was demonstrated in 2 different allergic inflammation models. In addition, CSE and nicotine inhibited allergen-induced Th2 cell responses *in vitro*.

The essential difference between the allergen-immunized and Th2 cell-transferred models is the contribution of allergen-specific immunoglobulins, including IgE. Mast cell activation via IgE cross-linking occurs in allergen-immunized mice but not in Th2-transferred mice following allergen challenge. Nevertheless, the effects of ETS on allergen-induced nasal responses were quite similar in both models. Thus, nasal eosinophil accumulation and NHR were significantly suppressed, whereas the migration of neutrophils was rather up-regulated. These findings are consistent with our previous investigation demonstrating the crucial contribution of CD4⁺ T cells to allergen-induced nasal inflammation [14]. Allergen-induced NHR accompanied by eosinophilic inflammation in allergen-immunized mice was substantially diminished by treatment with anti-CD4 neutralizing antibody, whereas the equivalent responses were developed in mast cell-deficient mice. Therefore, the suppression of allergen-induced nasal responses by ETS was most likely mediated, at least in part, by the inhibition of Th2-mediated responses.

The significant nasal accumulation of neutrophils observed in allergen-immunized but not in Th2-transferred mice suggests the pivotal contribution of IgE/mast cells in the immunization model, though neutrophil migration was augmented by ETS in both models. Consistent with this study, massive neutrophilia was found in COPD lung tissues in which ETS-mediated pathogenesis had been implicated [23]. Several mechanisms by which ETS induces airway neutrophilia have been investigated. For example, the production of the Th17-related cytokine IL-17 has been identified in COPD patients with increased neutrophil infiltration [24, 25]. ETS might facilitate Th17 differentiation *via* the up-regulation of IL-6 production by epithelial cells [26]. ETS contains a large amount of bioactive lipopolysaccharide [27]. Moreover, cigarette smoke delays spontaneous neutrophil death *via* the enhancement of Akt-mediated cell survival signals [28]. Although the contribution of various T cell subsets other than Th2 that produce chemoattractants for eosinophils and neutrophils deserves further investigation, it is important that the suppression of NHR by ETS was not caused by the augmentation of neutrophil migration into the nasal mucosa.

The allergen-induced proliferation of and IL-4 production by Th2 cells were suppressed by CSE and nicotine, suggesting that the down-regulation of Th2 cell responses was involved in the mechanisms by which ETS diminished allergen-induced nasal eosinophilia and NHR. Although ETS/CSE-mediated cytotoxic effects and nicotine-mediated pharmacological effects should be separately evaluated, it has been reported that the micromolar order concentration of nicotine was involved in 1% CSE [29]. These results are consistent with previous reports demonstrating that nicotine suppressed Th2 responses in allergic mouse lungs [30] and concanavalin-A-induced T cell proliferation in rats [31, 32]. It has been reported that current tobacco smokers have a low risk of developing allergic asthma and rhino-conjunctivitis [33]. Nizri et al. [34] reported that $\alpha 7$ nicotinic acetylcholine receptor (nAChR) signals display anti-inflammatory effects mediated by the reduction of NF- κ B transcription in T cells. $\alpha 4$ nAChR has also been implicated in the nicotine-mediated regulation of T cell functions [35]. Furthermore, T cells have the potential to produce acetylcholine [36]. Therefore, the contributions of these receptors to T cell responses and NHR pathogenesis require further study.

In summary, ETS suppressed allergen-induced nasal responses including NHR by inhibiting allergen-specific Th2 cell responses. Although our present findings do not deny harmful

effects of cigarette smoking, nicotine as a component of ETS may be a target to treat Th2-mediated allergic diseases, including AR.

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