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Original Article

Suppressive effect of dexamethasone on murine Th9 cell-mediated nasal eosinophilic inflammation

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Conflict of Interest
The authors have no financial conflicts of interest.

ABSTRACT

Background: Th9 cells have been implicated in the development of allergic inflammation, though its contribution to allergic rhinitis and the effect of steroid on Th9 cell-mediated nasal responses are unclear.
Objective: In this study, allergen-induced nasal inflammatory responses and their steroid responsiveness were investigated in ovalbumin (OVA)-specific Th9 cell-transferred mice.
Methods: BALB/c mice were transferred with *in vitro*-differentiated Th9 cells and challenged by intranasal injection of OVA with or without subcutaneous administration of dexamethasone (Dex). Then, the infiltration of inflammatory cells in the nasal mucosa and nasal hyperresponsiveness (NHR) was assessed.
Results: The significant NHR accompanied by nasal infiltration of eosinophils as well as allergen-specific T cells was induced in Th9 cell-transferred mice upon allergen challenge. These responses were strongly suppressed by the treatment with Dex.
Conclusion: The participation of Th9 cells in the pathogenesis of allergic rhinitis was suggested.

Keywords: Eosinophil; Mouse; Nasal hyperresponsiveness; Steroid; Th9 cell

INTRODUCTION

Inflammatory responses in the nasal mucosa are characteristic features of allergic rhinitis (AR) [1]. The nasal inflammation is frequently accompanied by the infiltration of eosinophils and T cells, and by the development of nasal hyperresponsiveness (NHR). Consequently, the exacerbation of nasal symptoms, such as sneezing, rhinorrhea, and nasal congestion is observed in AR patients [2].

Although mechanisms underlying allergic nasal inflammation are not fully been clarified, the pivotal role of $CD4^+$ T cells has been indicated. Nasal eosinophil accumulation as well as NHR induced by allergen challenge in immunized mice was suppressed by depleting $CD4^+$ T cells [3]. Allergen-induced nasal eosinophilia accompanied by significant NHR was evoked in normal mice by adoptive transfer of allergen-specific Th2 cells [3].

Author Contributions

Conceptualization: Osamu Kaminuma. Formal analysis: Kento Miura, Osamu Kaminuma. Investigation: Teidai Koyama, Norimasa Yamasaki, Sawako Ogata, Daiki Ito. Methodology: Mayumi Saeki, Akio Mori, Osamu Kaminuma. Project administration: Osamu Kaminuma. Writing - original draft: Teidai Koyama. Writing - review & editing: Kento Miura, Osamu Kaminuma.

Not only Th2 cells, but also many other $CD4^+$ T-cell subsets contribute to the development of allergic inflammation [4]. Among them, a character of Th9 cell originally identified as an interleukin (IL)-9-producing subset [5, 6] is similar, at least in part, to Th2 cell. Allergen-induced lung eosinophilia and bronchial hyperresponsiveness (BHR) could be induced in mice transferred with not only allergen-specific Th2 but also Th9 cells [7]. Interestingly, Th2- but not Th9-dependent BHR was suppressed by steroid treatment [8].

However, regardless of the existence in the nasal mucosa of AR patients [9], the potential of Th9 cells to induce allergic nasal inflammation has not been investigated. Therefore, we examined allergen-induced nasal accumulation of inflammatory cells and NHR in mice transferred with *in vitro*-differentiated Th9 cells. The effect of dexamethasone on the Th9 cell-dependent nasal inflammation model was further evaluated.

MATERIALS AND METHODS

Animals

Six-week-old female BALB/c mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan). DO11.10/RAG-$2^{-/-}$ mice were maintained for allergen-specific Th9 cell preparation as described previously [7]. The experimental protocols were approved by the Animal Use and Care Committee of Hiroshima University.

In vitro polarization of allergen-specific Th9 cells

Allergen-specific Th9 cells were prepared as described previously [7, 8]. Briefly, ovalbumin (OVA)-specific naïve $CD4^+$ T cells were isolated from splenocytes of DO11.10/RAG$2^{-/-}$ mice by positive selection using EasySep Mouse $CD4^+$ T Cell Isolation Kit (Veritas, Santa Clara, CA, USA). Cells were cultured with x-ray-irradiated splenocytes in AIM-V medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum. At the start of culture, 0.3-μM synthetic OVA323-339 peptide (Scrum Inc., Tokyo, Japan), 20-U/mL recombinant IL-2 (PeproTech, Rocky Hill, NJ, USA), 10-U/mL recombinant IL-4 (PeproTech), 5-ng/mL recombinant human TGF-β (BioLegend, San Diego, CA, USA), and 10-μg/mL anti-interferon-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. The successful differentiation of Th9 cells was confirmed elsewhere [7, 8].

Allergen-induced nasal responses

Polarized Th9 cells (2×10^7) were intravenously injected in each BALB/c mouse on day 0. From 6 hours after the cell transfer, these mice were challenged with intranasal administration of 20-μL OVA (30 mg/mL; Sigma, Sigma, St. Louis, MO, USA) or phosphate buffered saline (PBS) once a day on days 0–3 (**Fig. 1**). 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) [3]. Inflammatory cells in the nasal lavage fluid were classified by means of morphological criteria as described previously [3]. The number of transferred Th9 cells in nasal-associated lymphoid tissue (NALT) were determined by flow cytometry upon staining with anti-CD4-APC-Cy7 (BioLegend) and anti-DO11.10 T-cell receptor (TCR)-FITC (eBioscience). This procedure did not induce any inflammatory responses in the lower airways [10]. In some experiments, 10-mg/kg dexamethasone (Dex: Tokyo Kasei, Tokyo, Japan) suspended in PBS containing 0.5% Tween-20 (Sigma) was subcutaneously injected in mice 30 minutes before each OVA challenge.

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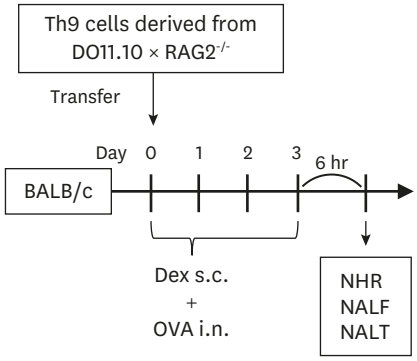

Fig. 1. Timeline of the experimental protocol. BALB/c mice transferred with *in vitro*-differentiated Th9 cells were subcutaneously (s.c.) administered with dexamethasone (Dex, 10 mg/kg) and challenged with intranasal injection (i.n.) of ovalbumin (OVA, 600 μg/head) once a day for 4 consecutive days. Six hours after the last challenge, the assessment of nasal hyperresponsiveness (NHR), nasal lavage fluid (NALF), and nasal-associated lymphoid tissue (NALT) was performed.

Statistical analysis

The results are presented as the arithmetic mean ± standard error of the mean. Normality of all quantitative data was analyzed by Shapiro-Wilk test prior to parametric or nonparametric test. As parametric test, the data were analyzed by 1-way analysis of variance followed by Dunnett test. As nonparametric test, the data were analyzed by Kruskal-Wallis test followed by Steel test. A p value of <0.05 was considered to indicate statistical significance.

RESULTS

Development of nasal inflammatory responses in Th9 cell-transferred mice

To evaluate the potency of Th9 cells to induce allergic nasal inflammation, allergen-specific Th9 cells were established from $CD4^+$ T cells of DO11.10/$RAG2^{-/-}$ mice in which OVA-reactive monoclonal TCR was expressed by *in vitro* stimulation culture as described in MATERIALS AND METHODS section. After confirming the adequate differentiation of the Th9 subset by evaluating cytokine production [7, 8], resulting cells were adoptively transferred to BALB/c mice, then those mice were challenged with intranasal administration of OVA (**Fig. 1**). Similar with the results for the Th2 cell transfer model previously reported [3], NHR (**Fig. 2**) and nasal eosinophil accumulation (**Fig. 3**) were significantly induced by the allergen challenge. Increases in lymphocytes, neutrophils, and macrophages in the NALT of allergen-challenged mice were also observed, though these responses were not statistically significant.

Effect of Dex on Th9 cell-mediated nasal inflammation

We previously demonstrated that allergic inflammation evoked by Th9 cells in the lung was resistant to steroid treatment [8]. Therefore, the effect of Dex on Th9 cell-mediated NHR and nasal cellular infiltration was examined. The allergen-induced NHR and migration of eosinophils in Th9 cell-transferred mice were significantly suppressed by the administration of Dex (**Figs. 2**, **3**).

Effect of Dex on Th9 cell migration

Next, the dynamics of allergen-specific Th9 cells in the draining lymphoid tissue was evaluated. By detecting DO11.10-TCR in flow cytometry, a significant increase in the infused

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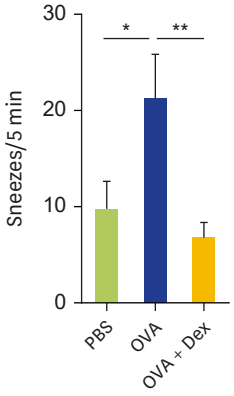

Fig. 2. Effect of dexamethasone (Dex) on allergen-induced nasal hyperresponsiveness (NHR) in Th9 cell-transferred mice. NHR was evaluated by counting the number of sneezes evoked by histamine as described in the MATERIALS AND METHODS section. Data are expressed as the mean ± standard error of the mean of 10–12 mice. PBS, phosphate buffered saline; OVA, ovalbumin. $^{*}p < 0.05$ and $^{**}p < 0.01$.

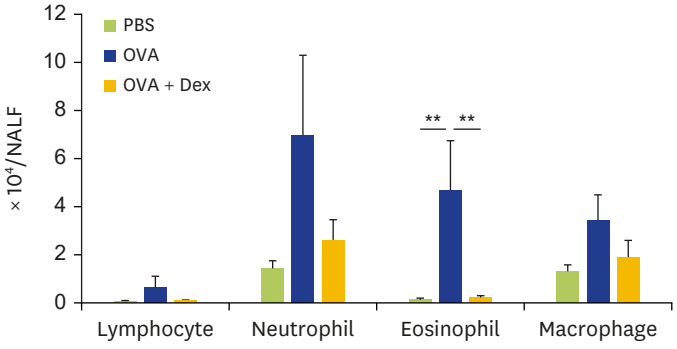

Fig. 3. Effect of dexamethasone (Dex) on allergen-induced nasal cellular infiltration in Th9 cell-transferred mice. The number of inflammatory cells in the nasal lavage fluid (NALF) was determined 6 hours after the last challenge. Data are expressed as the mean ± standard error of the mean of 9–12 mice. PBS, phosphate buffered saline; OVA, ovalbumin. $^{**}p < 0.01$.

Th9 cells in the NALT was observed upon allergen challenge. The allergen-induced migration of Th9 cells was significantly suppressed by Dex treatment (**Fig. 4A, B**).

DISCUSSION

Aside from the investigations regarding the role of Th9 cell in bronchial asthma, its contribution to the pathogenesis of AR, including NHR, has not sufficiently been examined. In this study, we demonstrated the significant development of allergic nasal inflammation with eosinophil infiltration and NHR in Th9 cell-transferred mice upon allergen challenge. In addition, the Th9 cell-mediated nasal responses were suppressed by steroid treatment.

By employing allergen-specific T-cell transfer model, we previously demonstrated that Th9 cells, like Th2 cells, have potential to induce eosinophilic inflammation in the lungs with significant BHR [7]. Our present results observed in the nasal mucosa are consistent with the previous findings and suggest the possible contribution of Th9 cells to allergic inflammation developed in various target organs.

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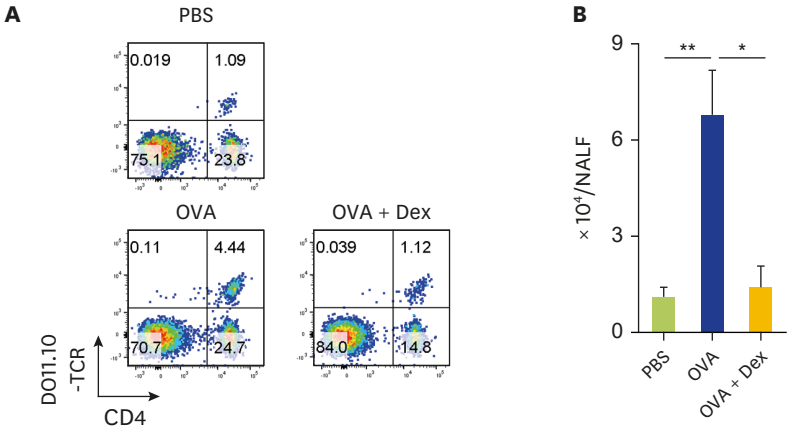

Fig. 4. Effect of dexamethasone (Dex) on allergen-induced migration of Th9 cells. The migration of allergen-specific cells in the nasal-associated lymphoid tissue (NALT) was determined by flow cytometry. The representative plots (A) and calculated data (B) are shown. Data are expressed as the mean ± standard error of the mean of 7 mice. PBS, phosphate buffered saline; OVA, ovalbumin; TCR, T-cell receptor. $^{*}p < 0.05$ and $^{**}p < 0.01$.

The essential difference between Th9 cell-mediated responses developed in the lungs and nasal mucosa is the responsiveness to steroid treatment. Contradictory to the present, allergen-induced BHR as well as lung accumulation of eosinophils and allergen-specific T cells in Th9 cell-transferred mice was not affected by the administration of Dex [8]. The reason for the discrepancy is currently unknown, though we have demonstrated that not only NHR but also BHR was suppressed by Dex when they were evoked in Th2 cell-transferred mice [8, 11]. Allergen-induced lung accumulation of Th2 cells but not Th9 cells was diminished by Dex [8]. Therefore, tissue specific surrounding environment responsible for Th9 cell migration including allergen-presenting cells, chemokines, and adhesion molecules may decide the steroid responsiveness of Th9 cell-mediated responses in each tissue.

The mechanisms underlying Th9 cell-mediated NHR are unclear. Regardless of the preferential accumulation of eosinophils or neutrophils in the nasal mucosa, we have shown that not only Th2 cells but also Th1 and Th17 cells could induce NHR by adoptive transfer [3]. Eosinophils accumulated in the lungs crucially contribute to the development of allergen-induced BHR in Th2 but not Th9 cell-transferred mice [7]. A treatment with IL-9-neutralizing antibody did not affect Th9 cell-mediated BHR [7]. The evaluation of eosinophil-dependency and identification of responsible molecules in Th9 cell-mediated NHR deserve further investigation.

In summary, Th9 cells have potential to induce allergen-induced nasal responses including NHR and accumulation of eosinophils and allergen-specific T cells. The efficacy of steroids to AR patients may involve down-regulation of Th9 cell-mediated responses. Possible management of AR by targeting Th9-mediated nasal inflammation is suggested.

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