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Original article

Th9 cells elicit eosinophil-independent bronchial hyperresponsiveness in mice

Mayumi Saeki [a, *], Osamu Kaminuma [a], Tomoe Nishimura [a], Noriko Kitamura [a], Akio Mori [b], Takachika Hiroi [a]

[a] Allergy and Immunology Project, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan
[b] Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagamihara National Hospital, Kanagawa, Japan

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Abbreviations:
Ab, antibody; BALF, bronchoalveolar lavage fluid; BHR, bronchial hyperresponsiveness; H&E, hematoxylin and eosin; i.v., intravenous; MCh, methacholine; OVA, ovalbumin; PAS, periodic acid-Schiff; Rrs, respiratory system resistance; SEM, standard error of mean; TGF-β, transforming growth factor-β; WT, wild-type

ABSTRACT

Background: Airway accumulation of eosinophils and bronchial hyperresponsiveness (BHR) are prominent features of bronchial asthma, though the contribution of eosinophils to the development of BHR is controversial. Similar to Th2 cell-mediated pathology, Th9 cells, characterized by IL-9-producing activity, have been demonstrated to induce airway eosinophilia and BHR. In this study, we investigated the role of eosinophils in Th9-mediated BHR by employing Th9 cell-transferred murine airway inflammation model.
Methods: Ovalbumin (OVA)-specific Th2 and Th9 cells were differentiated from $CD4^+$ T cells of DO11.10/*RAG-2*$^{-/-}$ mice *in vitro* and cytokine-producing activity of those cells was examined. BALB/c mice were adoptively transferred with Th2 or Th9 cells and challenged with OVA. Then, the number of inflammatory cells in bronchoalveolar lavage fluid and bronchial responsiveness to inhaled methacholine were determined.
Results: Both in Th2 and Th9 cell-transferred mice, substantial accumulation of eosinophils in the lungs and BHR were induced by challenge with specific antigen. Nevertheless, an essential and dispensable role of eosinophils in Th2- and Th9-mediated BHR, respectively, was demonstrated by employing eosinophil-deficient mice. The neutralization of IL-9 as well as deficiency of IL-10 in the donor cells did not affect Th9-mediated BHR.
Conclusions: In contrast to Th2-mediated and eosinophil-dependent BHR, Th9 could induce BHR independently from eosinophils and its characteristic cytokines, IL-9 and IL-10.

Introduction

Bronchial asthma is a chronic inflammatory disease characterized by reversible airway obstruction and bronchial hyperresponsiveness (BHR) associated with eosinophilic inflammation. Th2 cells have been recognized to regulate pathological features of this disease by secreting cytokines such as IL-4, IL-5, and IL-13.[1–4] These cytokines induce a variety of responses including IgE production as well as airway eosinophilia, mucus production, and remodeling. Human asthma-like Th2-type airway inflammation could be reproduced in mouse models. Thus, antigen-induced eosinophil accumulation in the lungs accompanied by significant BHR was observed in mice transferred with *in vitro*-differentiated antigen-specific Th2 cells.[5,6]

Activated eosinophils have been implicated in asthma pathogenesis. It has been recognized that BHR is mediated by accumulated eosinophils in the lungs of asthmatic patients.[7] However, recent conflicting findings in several clinical studies using neutralizing antibodies (Abs) against eosinophil-active cytokines have unstabilized the contribution of eosinophils to BHR.[8–11] Such controversy has been seen also in animal studies.[12]

We have also investigated the contribution of eosinophils to BHR, especially in a Th2-mediated murine airway inflammation model. Thus, using eosinophil-deficient mice in which double GATA site in the *GATA-1* gene was deleted (ΔdblGATA) as the recipients,[13,14] attenuated antigen-induced BHR was observed. In

* Corresponding author. Allergy and Immunology Project, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan.
E-mail address: saeki-my@igakuken.or.jp (M. Saeki).
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addition, intervention of CCR3, a chemokine receptor dominantly expressed on eosinophils, significantly suppressed Th2-mediated BHR along with the reduction of airway eosinophil accumulation.[5] These findings support the critical role of accumulated eosinophils induced by Th2 cells in the development of BHR.

In addition to well-known T cell subsets such as Th1, Th2, and Th17, a new subset characterized by IL-9-producing activity has recently been identified and termed as Th9 cells.[15,16] Th9 cells are developed from naive $CD4^+$ T cells by priming with IL-4 and transforming growth factor-β (TGF-β). Similar to Th2 cell-mediated models, mice transferred with Th9 cells develop asthma-like airway inflammation characterized by eosinophil infiltration and BHR in response to airway antigen challenge.[17] In addition, IL-9 induces the expression of mucus genes in airway epithelial cells.[18–21] Therefore, Th9 cell is recognized as one of the key players and a target for the treatment of bronchial asthma.[22,23]

However, also in the case of Th9-mediated pathogenesis, it has not been determined whether eosinophils play a role in the development of BHR. Here, we investigated the mechanisms of Th9 cell-mediated BHR including the contribution of eosinophils by employing eosinophil-deficient mice, anti-IL-9 neutralizing Ab, and IL-10-deficient Th9 cells in the Th9 cell-transferred murine model of airway inflammation.

Methods

Experimental animals

All animal experiments were performed in accordance with guidelines approved by the animal use committee at Tokyo Metropolitan Institute of Medical Science. DO11.10/*RAG-2*$^{-/-}$, IL-$10^{-/-}$/DO11.10/*RAG-2*$^{-/-}$, and ΔdblGATA mice of BALB/c background were maintained as described previously.[6,14,24]

In vitro T cell differentiation

After depletion of erythrocytes, $CD4^+$ T cells were isolated from splenocytes of DO11.10/*RAG-2*$^{-/-}$ and IL-$10^{-/-}$/DO11.10/*RAG-2*$^{-/-}$ mice by using anti-mouse CD4 Ab-conjugated magnetic beads and magnetic cell sorting system (Miltenyi, Bergisch Gladbach, Germany). Then, cells were cultured in the presence of X-ray-irradiated syngeneic spleen cells as antigen-presenting cells and 0.3 μM OVA323-339 peptide in DMEM-F12/HAM medium (Sigma-Aldrich, MO, USA) supplemented with 10% fetal bovine serum, penicillin, streptomycin, L-glutamine, HEPES, pyruvate, and 2-mercaptoethanol. Th2 differentiation was introduced by adding 10 U/ml recombinant human IL-2 (Shionogi, Osaka, Japan) and mouse IL-4 (PeproTech, NJ, USA) and 10 μg/ml anti-IFN-γ monoclonal Ab (mAb) (R4-6A2, eBioscience, CA, USA) as described previously.[6,24] Th9 cells were differentiated by adding 10 U/ml IL-2 and IL-4, 5 ng/ml recombinant mouse TGF-β (R&D Systems, MN, USA), and 10 μg/ml anti-IFN-γ mAb. Cells were cultured for 7 days and then used for the adoptive transfer. To determine the integrity of polarization, cells (1×10^5) were incubated with irradiated splenocytes (2×10^5) with or without 0.3 μM OVA peptide for 72 h. The culture supernatants were collected to determine cytokine production by ELISA using mouse ELISA kits (eBioscience and BioLegend, CA, USA) according to the manufacturer's instructions.

Cell transfer and antigen challenge

Cells (1×10^7) suspended in phosphate-buffered saline were intravenously (i.v.) injected in the recipient mice. One day after the cell transfer, the mice were challenged with intratracheal injection of OVA solution (25 μL, 15 mg/ml in saline) using a MicroSprayer aerosolizer (Penn Century Inc., PA, USA) under isoflurane anesthesia as described previously.[25] To neutralize IL-9, mice were administered with either 6 mg/kg (i.v.) anti-IL-9 (BioLegend, #504802) or isotype control Ab 2 h before the OVA challenge.

Bronchoalveolar lavage fluid analysis

Seventy-two hours after the antigen challenge, bronchoalveolar lavage was performed by introducing 3 × 0.5 ml phosphate-buffered saline into the lung *via* a tracheal cannula. The number of leukocytes in bronchoalveolar lavage fluid (BALF) was counted using a hemocytometer. Then, differential cell counts based on morphologic criteria were performed for at least 200 cells on a cytocentrifuged preparation after staining with Diff-Quick (Sysmex Corporation, Kobe, Japan). The number of transferred T cells in BALF was determined by flow cytometry upon staining with anti-CD4-APC-eFluor780 (eBioscience) and anti-KJ1-26-PE (BioLegend).

Measurement of bronchial hyperresponsiveness

Seventy-two hours after the antigen challenge, mice were anesthetized by intraperitoneal injection of 100 mg/kg sodium pentobarbital (Kyoritsu Seiyaku, Tokyo, Japan) and then a 19-gauge cannula was inserted into the trachea. Mechanical ventilation was performed under diaphragmatic perforation using a small animal ventilator (FlexiVent; SCIREQ, Quebec, Canada) at a respiratory rate of 150 breaths/min, a tidal volume of 10 ml/kg body weight, and a positive end expiratory pressure of 3 cmH_2O. BHR was assessed by measuring progressive change in respiratory system resistance (Rrs) following inhalation of increasing doses of aerosolized methacholine (MCh; Nacalai tesque, Kyoto, Japan) through an in-line nebulizer.

Lung histology

Lung tissues were fixed in 4% paraformaldehyde, and embedded in paraffin. Tissue sections (4 μm) were stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) by the standard procedure.

Statistical analysis

The results are presented as arithmetic mean ± standard error of mean (SEM). Statistical analysis was performed using Student's *t*-test or one-way analysis of variance and Dunnett's multiple comparison test. *p* values less than 0.05 were considered to indicate statistical significance.

Table 1
Cytokine production by Th2 and Th9 cells.

	Concentration (ng/ml)		*p* value
	Th2	Th9	
IL-4	611.4 ± 20.1	118.2 ± 1.5	0.002
IL-5	45.1 ± 3.1	16.4 ± 0.1	0.012
IL-9	1.9 ± 1.0	9.2 ± 1.0	0.006
IL-10	156.5 ± 36.9	85.5 ± 1.7	0.195

In vitro-differentiated Th2 and Th9 cells were cultured with X-ray irradiated splenocytes in the presence of OVA323-339 peptide. Seventy-two hours later, the concentrations of cytokines in the culture supernatants were measured by ELISA. Data are expressed as mean ± SEM of triplicate cultures. The results shown are representatives of three separate experiments.