



Contents lists available at ScienceDirect

Allergy International

journal homepage: <http://www.elsevier.com/locate/alit>

Original article

Th9 cells elicit eosinophil-independent bronchial hyperresponsiveness in mice

Mayumi Saeki^{a,*}, Osamu Kaminum^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, Sagami National Hospital, Kanagawa, Japan

ARTICLE INFO

Article history:

Received 29 January 2016

Received in revised form

15 April 2016

Accepted 5 May 2016

Available online xxx

Keywords:

Bronchial asthma

Bronchial hyperresponsiveness

Eosinophil

Eosinophil-deficient mice

Th9 cells

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.

Copyright © 2016, Japanese Society of Allergy. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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 (Δ dbGATA) 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).

Peer review under responsibility of Japanese Society of Allergy.

<http://dx.doi.org/10.1016/j.alit.2016.05.003>

1323–8930/Copyright © 2016, Japanese Society of Allergy. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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 Δ dblGATA 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 μ M 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 μ g/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 μ g/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 μ M 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 μ L, 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 cmH₂O. 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 μ m) 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 $\pm$ 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)		<i>p</i> value
	Th2	Th9	
IL-4	611.4 $\pm$ 20.1	118.2 $\pm$ 1.5	0.002
IL-5	45.1 $\pm$ 3.1	16.4 $\pm$ 0.1	0.012
IL-9	1.9 $\pm$ 1.0	9.2 $\pm$ 1.0	0.006
IL-10	156.5 $\pm$ 36.9	85.5 $\pm$ 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 $\pm$ SEM of triplicate cultures. The results shown are representatives of three separate experiments.

Results

Characterization of differentiated T cells *in vitro*

To evaluate the phenotypes of *in vitro*-differentiated Th2 and Th9 cells, their cytokine-secreting profiles were examined. CD4⁺ T cells differentiated into Th2 cells produced IL-4, IL-5, and IL-10, and a small amount of IL-9 upon stimulation with OVA (Table 1). In contrast, the cells grown under the Th9 conditions produced substantial amounts of IL-9 and IL-10 though their IL-4- and IL-5-producing activity were significantly lower than Th2 cells. Detectable amounts of those cytokines were not produced by Th2 and Th9 cells without antigen stimulation (data not shown), suggesting that the typical Th2 and Th9 cells were developed under the appropriate culture conditions *in vitro*.

Antigen-induced airway inflammation in Th2 and Th9 cell-transferred wild-type and eosinophil-deficient mice

The infiltration of inflammatory cells into the lungs was determined in Th2 or Th9 cell-transferred mice. Large numbers of eosinophils, lymphocytes, and neutrophils were recovered in BALF of antigen-challenged both mice (Fig. 1A, C). The bronchial responsiveness to inhaled MCh was similarly augmented in Th2- and Th9-transferred mice by OVA challenge (Fig. 1B, D). Antigen-specific immunoglobulins were not produced in this short-term experimental procedure (data not shown). These results are consistent with previous reports showing the critical role of Th2 and Th9 cells in the development of eosinophilic inflammation accompanied by BHR^{14,26} and suggest that Th2 and Th9 cells enable to induce airway eosinophil accumulation and BHR by themselves without assistance of IgE and other antigen-specific immunoglobulins.

Next, to examine the contribution of eosinophils to BHR induced by Th2 and Th9 cells, these cells were transferred to Δ dblGATA mice. Although eosinophils were not detectable in BALF of Th2- and Th9-transferred Δ dblGATA mice even after OVA challenge, there was no major difference in the infiltration of other inflammatory cells between wild-type (WT) and Δ dblGATA mice (Fig. 1A, C).

Nevertheless, the role of eosinophils in BHR was different in Th2- and Th9-mediated airway inflammation. In agreement with our previous study,¹⁴ antigen-induced increase in bronchial responsiveness was not observed in Th2 cell-transferred Δ dblGATA mice (Fig. 1B). Nevertheless, the equivalent antigen-induced BHR was developed in WT and Δ dblGATA mice by using Th9 as donor cells (Fig. 1D).

H&E staining of the lungs illustrated that the same level perivascular and peribronchial inflammation was developed in Th2- and Th9-transferred WT and Δ dblGATA mice after OVA challenge even without the infiltration of eosinophils (Fig. 1E). Antigen-induced hyperplasia of PAS-positive goblet cells was also similarly observed in the airway epithelium of those mice. These data suggest that Th9 cell-mediated BHR is not dependent on eosinophils accumulated in the lungs, in contrast to eosinophil-dependent BHR induced by Th2 cells.

To investigate the direct contribution of antigen-specific T cells to the development of BHR, we next examined OVA-induced migration of transferred T cells in the lungs. The non-specific distribution of Th9 cells was observed in saline-challenged WT mice to some degree. The significant infiltration of infused T cells was induced in Th2-transferred mice by OVA challenge, though it was more efficiently induced in Th9-transferred mice (Fig. 1F).

Role of IL-9 and IL-10 in Th9 cell-mediated BHR

IL-9 and IL-10 produced by activated Th9 cells have been implicated in asthma pathogenesis,^{22,23} therefore the contribution

of these cytokines to the development of Th9-mediated BHR was evaluated. Th9 cell-transferred Δ dblGATA mice were challenged with OVA after the treatment with anti-IL-9 Ab. There was no major difference in antigen-induced BHR between anti-IL-9- and control IgG-treated mice (Fig. 2). Two other anti-IL-9 Abs also failed to affect Th9-mediated BHR (data not shown).

Th9 cells established from IL-10^{-/-}/DO11.10/RAG-2^{-/-} mice were transferred to WT and Δ dblGATA mice, and then these mice were challenged with OVA. Essentially same as the case that IL-10^{+/+} Th9 cells were used as donor cells (Fig. 1A), the equivalent infiltration of inflammatory cells except for eosinophils was observed in the lungs of Δ dblGATA and WT mice (Fig. 3A). Antigen-induced BHR was rather augmented by IL-10 deficiency in donor Th9 cells but was not affected by using Δ dblGATA mice as the recipient (Fig. 3B). These data suggest that the development of BHR was not caused by IL-9 and IL-10 produced from Th9 cells.

Discussion

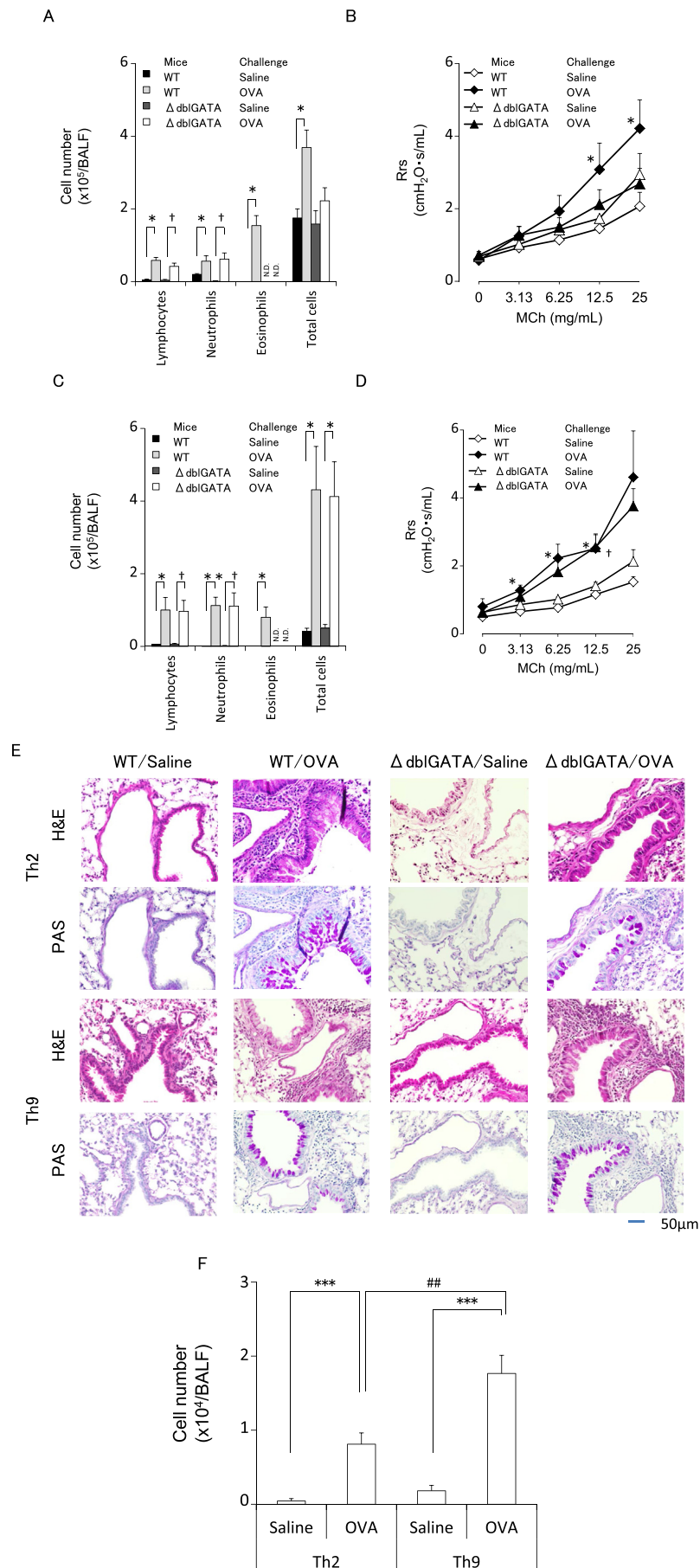
Although inflammatory features including eosinophil accumulation and BHR shown in Th2- and Th9-mediated airway inflammation quite resemble each other, whether the same mechanisms underlie the both responses has not been clarified. In this study, we elucidated that Th9 cell-mediated BHR, in contrast to Th2-mediated response, was not dependent on eosinophils infiltrated in the lungs.

Several attempts to treat bronchial asthma through the intervention of eosinophil-active cytokines have been performed. However, clinical studies demonstrating the decreased sputum eosinophils but unchanged BHR by the treatment with anti-IL-5 Ab made the contribution of eosinophils to BHR doubtful.^{9–11} Considering our present findings showing the independency of Th9-mediated BHR from eosinophils, the efficacy of such eosinophil-targeted therapy might not be expected for some asthma endotypes in which the participation of Th9 cells is relatively high. Since it has recently been elucidated that anti-IL-5 therapy is effective for steroid-dependent asthmatic patients,^{27,28} down-regulation of Th9 activity might be induced by the steroid treatment in those patients.

The mechanisms by which Th9 cells induced BHR without assistance of eosinophils were not completely evaluated in this study. However, several cytokines such as IL-13, IL-17A, and TNF- α directly regulate BHR by increasing calcium sensitivity of airway smooth muscle cells through signaling pathways involving NF- κ B, RhoA, and ROCK2.^{29–32} Here we showed that Th9 cells more efficiently migrated in the lungs than Th2 cells upon antigen provocation. Therefore, Th9 may directly affect the contractile activity of airway smooth muscle by secreting its specific cytokines.

In this regard, Th9 cells predominantly secrete IL-9 and IL-10. IL-10 modulates many cell activities that are implicated in allergic diseases. Increased IL-10 in the lungs could diminish allergic airway inflammation.³³ Repeated airway antigen exposure leads tolerance *via* the induction of IL-10-secreting regulatory T cells.³⁴ Paradoxically, Justice *et al.* and Mäkelä *et al.* showed that antigen-induced BHR was attenuated by IL-10 gene disruption.^{35,36} Our present findings that Th9-mediated BHR was not suppressed but rather augmented by deficiency of IL-10 support the negative role of IL-10 in the development of airway inflammation.

The essential contribution of IL-9 to Th9-mediated BHR was also dismissed by its neutralizing experiment. However, our findings are contradictory to the report of Staudt *et al.* demonstrating the down-regulation of BHR by anti-IL-9 Ab.²⁶ Although the correct reason for the discrepancy was unclear, Staudt *et al.* used RAG-2^{-/-} mice as the Th9 cell recipients and challenged antigen for 6 consecutive days. The chronic antigen exposure might elevate the dependency of BHR on IL-9. Further investigation with beware the differences in



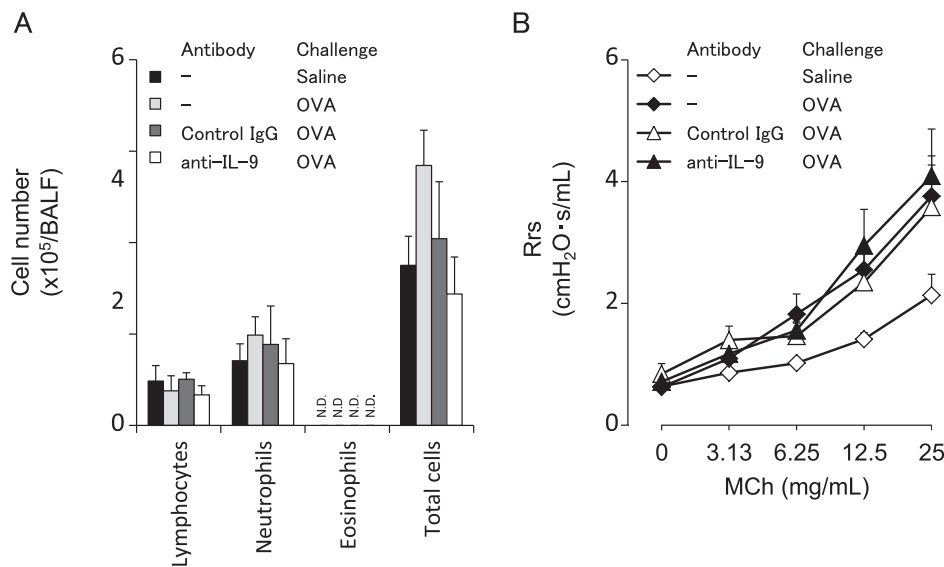


Fig. 2. Effect of anti-IL-9 Ab on Th9-mediated BHR. Th9 cell-transferred Δ dblGATA mice were challenged with OVA or saline. Seventy-two hours after the challenge, inflammatory cells recovered in BALF were differentially counted (A) and bronchial responsiveness to inhaled MCh was assessed (B). Data are expressed as mean $\pm$ SEM of 3–7 animals. N.D., not detectable.

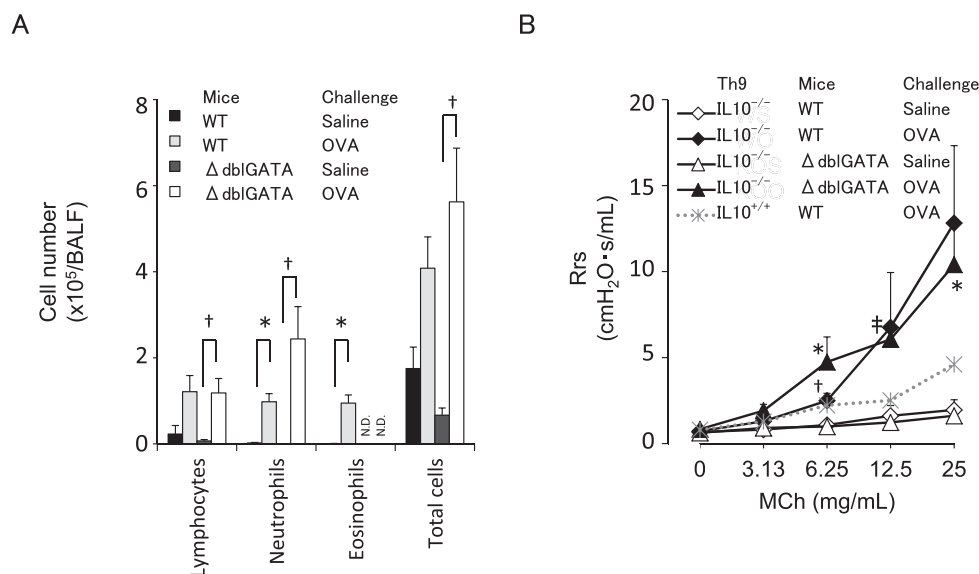


Fig. 3. Antigen-induced airway inflammation in IL-10-deficient Th9 cell-transferred mice. IL-10^{-/-} Th9 cell-transferred WT and Δ dblGATA mice were challenged with OVA or saline. Seventy-two hours after the challenge, inflammatory cells recovered in BALF were differentially counted (A) and bronchial responsiveness to inhaled MCh was assessed (B). The data of IL-10^{+/+} Th9 cell-transferred and antigen-challenged WT mice shown in Fig. 1D were also inset for the comparison (B). Data are expressed as mean $\pm$ SEM of 3–7 animals. * p < 0.05, compared with saline-challenged WT mice. † p < 0.05, ‡ p < 0.01, compared with saline-challenged Δ dblGATA mice. N.D., not detectable.

experimental conditions employed by Staudt *et al.* and us may be required to elucidate the exact role of IL-9 in the development of BHR.

In addition to IL-9 and IL-10, the expression of several genes encoding inflammatory mediators was recently identified in Th9

cells by microarray analysis.³⁷ Therefore, unknown mediators might play a role in BHR induced by Th9 cells.

In conclusion, unlikely to Th2-mediated response, Th9-mediated BHR was independent of eosinophils accumulated into the lungs. The elucidation of the mechanisms underlying Th9-

Fig. 1. Antigen-induced airway inflammation in Th2 and Th9 cell-transferred mice. Th2 (A, B) or Th9 (C, D) cell-transferred WT and Δ dblGATA mice were challenged with OVA or saline. Seventy-two hours after the challenge, inflammatory cells recovered in BALF were differentially counted (A, C) and bronchial responsiveness to inhaled MCh was assessed (B, D). H&E-stained (upper panels) or PAS-stained (lower panels) lung sections were observed under microscopy (E). The number of infused T cells recovered in BALF of Th2 and Th9 cell-transferred WT mice was examined by flow cytometry (F). Data are expressed as mean $\pm$ SEM of 3–10 animals. * p < 0.05, ** p < 0.01, *** p < 0.001, compared with saline-challenged WT mice. † p < 0.05, ‡ p < 0.01, compared with saline-challenged Δ dblGATA mice. ## p < 0.01, compared with antigen-challenged Th2 cell-transferred mice. N.D., not detectable.

mediated BHR is favorable to the development of a novel therapeutic strategy for bronchial asthma.

Acknowledgments

This work was supported by Grant-in-Aid for JSPS KAKENHI (No. 24791817 and 26860122) to M. Saeki and Grant-in-Aid for JSPS KAKENHI (No. 15K07787), the Astellas Foundation for Research on Metabolic Disorders (No. 1090), the Towa Foundation for Food Science and Research, the Kieikai Research Foundation, the Hoya Science Foundation (No. 22), Skylark Food Science Institute Foundation, and the Smoking Research Foundation to O. Kaminuma.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

MS designed the study and wrote the manuscript. OK supervised this work and wrote the manuscript. TN and NK contributed to data collection. AM and TH contributed to editing of the paper. All authors read and approved the final manuscript.

References

- Wills-Karp M. Immunological basis of antigen-induced airways hyper-responsiveness. *Annu Rev Immunol* 1999;17:255–81.
- Kay AB. Asthma and inflammation. *J Allergy Clin Immunol* 1991;87:893–910.
- Bochner BS, Undem BJ, Lichtenstein LM. Immunological aspects of allergic asthma. *Annu Rev Immunol* 1994;12:295–335.
- Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, et al. Predominant TH2-like bronchoalveolar lavage T-lymphocyte population in atopic asthma. *N Engl J Med* 1992;326:298–304.
- Mori A, Ogawa K, Someya K, Kunori Y, Nagakubo D, Yoshie O, et al. Selective suppression of Th2-mediated airway eosinophil infiltration by low-molecular weight CCR3 antagonists. *Int Immunol* 2007;19:913–21.
- Kaminuma O, Ohtomo T, Mori A, Nagakubo D, Hieshima K, Ohmachi Y, et al. Selective downregulation of Th2 cell-mediated airway inflammation in mice by pharmacological intervention of CCR4. *Clin Exp Allergy* 2012;42:315–25.
- Kraneveld AD, Folkerts G, Van Oosterhout AJ, Nijkamp FP. Airway hyper-responsiveness: first eosinophils and then neuropeptides. *Int J Immunopharmacol* 1997;19:517–27.
- Simon D, Braathen LR, Simon HU. Anti-Interleukin-5 antibody therapy in eosinophilic diseases. *Pathobiology* 2005;72:287–92.
- Bochner BS, Gleich GJ. What targeting eosinophils has taught us about their role in diseases. *J Allergy Clin Immunol* 2010;126:16–25.
- Garcia G, Taillé C, Laveneziana P, Bourdin A, Chanez P, Humbert M. Anti-interleukin-5 therapy in severe asthma. *Eur Respir Rev* 2013;22:251–7.
- Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, et al. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 2000;356:2144–8.
- Kay AB. The role of eosinophils in the pathogenesis of asthma. *Trends Mol Med* 2005;11:148–52.
- Yu C, Cantor AB, Yang H, Browne C, Wells RA, Fujiwara Y, et al. Targeted deletion of a high-affinity GATA-binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage in vivo. *J Exp Med* 2002;195:1387–95.
- Ohtomo T, Kaminuma O, Yamada J, Kitamura N, Abe A, Kobayashi N, et al. Eosinophils are required for the induction of bronchial hyperresponsiveness in a Th transfer model of BALB/c background. *Int Arch Allergy Immunol* 2010;152(Suppl 1):79–82.
- Dardalhon V, Awasthi A, Kwon H, Galileos G, Gao W, Sobel RA, et al. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat Immunol* 2008;9:1347–55.
- Veldhoen M, Uytendhoeve C, van Snick J, Helmby H, Westendorp A, Buer J, et al. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol* 2008;9:1341–6.
- Goswami R, Kaplan MH. A brief history of IL-9. *J Immunol* 2011;186:3283–8.
- Longphre M, Li D, Gallup M, Drori E, Ordoñez CL, Redman T, et al. Allergen-induced IL-9 directly stimulates mucin transcription in respiratory epithelial cells. *J Clin Invest* 1999;104:1375–82.
- Louahed J, Toda M, Jen J, Hamid Q, Renaud JC, Levitt RC, et al. Interleukin-9 upregulates mucus expression in the airways. *Am J Respir Cell Mol Biol* 2000;22:649–56.
- Reader JR, Hyde DM, Schelegle ES, Aldrich MC, Stoddard AM, McLane MP, et al. Interleukin-9 induces mucous cell metaplasia independent of inflammation. *Am J Respir Cell Mol Biol* 2003;28:664–72.
- Vermee PD, Harson R, Einwalter LA, Moninger T, Zabner J. Interleukin-9 induces goblet cell hyperplasia during repair of human airway epithelia. *Am J Respir Cell Mol Biol* 2003;28:286–95.
- Vock C, Hauber HP, Wegmann M. The other T helper cells in asthma pathogenesis. *J Allergy* 2010;2010:519298.
- Kaplan MH, Hufford MM, Olson MR. The development and in vivo function of T helper 9 cells. *Nat Rev Immunol* 2015;15:295–307.
- Nishimura T, Saeki M, Motoi Y, Kitamura N, Mori A, Kaminuma O, et al. Selective suppression of Th2 cell-mediated lung eosinophilic inflammation by anti-major facilitator super family domain containing 10 monoclonal antibody. *Allergol Int* 2014;63(Suppl 1):29–35.
- Saeki M, Nishimura T, Mori A, Kaminuma O, Hiroi T. Antigen-induced mixed and separated inflammation in murine upper and lower airways. *Allergol Int* 2014;63(Suppl 1):59–61.
- Staudt V, Bothur E, Klein M, Lingnau K, Reuter S, Grebe N, et al. Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity* 2010;33:192–202.
- Castro M, Wenzel SE, Bleecker ER, Pizzichini E, Kuna P, Busse WW, et al. Benralizumab, an anti-interleukin 5 receptor α monoclonal antibody, versus placebo for uncontrolled eosinophilic asthma: a phase 2b randomised dose-ranging study. *Lancet Respir Med* 2014;2:879–90.
- Castro M, Zangrilli J, Wechsler ME, Bateman ED, Brusselle GG, Bardin P, et al. Reslizumab for inadequately controlled asthma with elevated blood eosinophil counts: results from two multicentre, parallel, double-blind, randomised, placebo-controlled, phase 3 trials. *Lancet Respir Med* 2015;3:355–66.
- Chiba Y, Nakazawa S, Todoroki M, Shinzaki K, Sakai H, Misawa M. Interleukin-13 augments bronchial smooth muscle contractility with an up-regulation of RhoA protein. *Am J Respir Cell Mol Biol* 2009;40:159–67.
- Goto K, Chiba Y, Matsusue K, Hattori Y, Maitani Y, Sakai H, et al. The proximal STAT6 and NF-kappaB sites are responsible for IL-13- and TNF-alpha-induced RhoA transcriptions in human bronchial smooth muscle cells. *Pharmacol Res* 2010;61:466–72.
- Kudo M, Melton AC, Chen C, Engler MB, Huang KE, Ren X, et al. IL-17A produced by $\alpha\beta$ T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction. *Nat Med* 2012;18:547–54.
- Chen H, Tliba O, Van Besien CR, Panettieri Jr RA, Amrani Y. TNF-alpha modulates murine tracheal rings responsiveness to G-protein-coupled receptor agonists and KCl. *J Appl Physiol* (1985) 2003;95:864–72 (discussion 863).
- Stämpfli MR, Cwiartka M, Gajewska BU, Alvarez D, Ritz SA, Inman MD, et al. Interleukin-10 gene transfer to the airway regulates allergic mucosal sensitization in mice. *Am J Respir Cell Mol Biol* 1999;21:586–96.
- Akbari O, Freeman GJ, Meyer EH, Greenfield EA, Chang TT, Sharpe AH, et al. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat Med* 2002;8:1024–32.
- Mäkelä MJ, Kanehiro A, Borish L, Dakhama A, Loader J, Joetham A, et al. IL-10 is necessary for the expression of airway hyperresponsiveness but not pulmonary inflammation after allergic sensitization. *Proc Natl Acad Sci USA* 2000;97:6007–12.
- Justice JP, Shibata Y, Sur S, Mustafa J, Fan M, Van Scott MR. IL-10 gene knockout attenuates allergen-induced airway hyperresponsiveness in C57BL/6 mice. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L363–8.
- Jabeen R, Goswami R, Awe O, Kulkarni A, Nguyen ET, Attenasio A, et al. Th9 cell development requires a BATF-regulated transcriptional network. *J Clin Invest* 2013;123:4641–53.