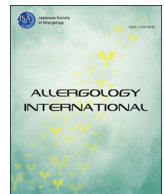




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Original article

Th9 cells induce steroid-resistant bronchial hyperresponsiveness in mice

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Ab, antibody; BALF, bronchoalveolar lavage fluid; BHR, bronchial hyperresponsiveness; Dex, dexamethasone; GR, glucocorticoid receptor; IFN- γ , interferon-gamma; IL, interleukin; MCh, methacholine; OVA, ovalbumin; PBS, phosphate-buffered saline; Rrs, respiratory system resistance; SEM, standard error of mean; TGF- β , transforming growth factor-beta

ABSTRACT

Background: Reduced responsiveness to corticosteroid therapy is a major problem for patients with severe asthma. Although Th9 cells, along with Th2 cells, facilitate antigen-induced airway eosinophilia and bronchial hyperresponsiveness (BHR), the sensitivity of Th9 cell-mediated responses to steroid therapy remains unknown. In this study, we investigated the effect of dexamethasone (Dex) on antigen-induced airway inflammation in Th9 cell-transferred mice.

Methods: Ovalbumin (OVA)-specific Th2 and Th9 cells were polarized from the CD4⁺ T cells of DO11.10/RAG-2^{-/-} mice. BALB/c mice were adoptively transferred with Th2 or Th9 cells and challenged with OVA. Dex treatment was performed twice, at 1 h before and at 24 h after the OVA challenge. Following treatment, the number of inflammatory cells in the bronchoalveolar lavage fluid and the bronchial responsiveness to inhaled methacholine were determined.

Results: In both the Th2 and Th9 cell-transferred mice, substantial accumulation of eosinophils in the lungs and BHR were induced by challenge with the specific antigen. In the Th2 cell-transferred mice, these responses were significantly diminished by Dex treatment. In contrast, neither cellular infiltration nor BHR was affected by Dex treatment in the Th9 cell-transferred mice, although the Th9 cells substantially expressed glucocorticoid receptor α . Accordingly, antigen-induced interleukin-9 expression in the Th9 cells was attenuated by Dex treatment at least *in vitro*. Antigen-induced lung infiltration of infused Th2 cells but not Th9 cells was significantly suppressed by Dex.

Conclusions: In contrast to Th2-mediated responses, Th9-mediated airway inflammation was not affected by Dex. Th9 cells might be involved in the developmental mechanisms of steroid-resistant asthma.

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Introduction

Bronchial asthma is a chronic inflammatory disease. Typically, asthma is characterized by reversible airway obstruction, mucus production, and bronchial hyperresponsiveness (BHR) associated with eosinophilic inflammation.^{1,2} Th2 cells have been recognized to play a role in the pathogenesis of asthma by secreting cytokines such as interleukin (IL)-4, IL-5, and IL-13.^{1–4} These cytokines induce various responses, including immunoglobulin E production, airway

eosinophilia, and mucus production.^{1–4} Human asthma-like Th2-type airway inflammation could be reconstituted in mouse adoptive transfer models. Thus, antigen-induced eosinophil accumulation in the lungs accompanied by significant BHR has been observed in mice transferred with *in vitro*-differentiated antigen-specific Th2 cells.^{5,6}

Th9 cells are a new subset of CD4⁺ T cells characterized by IL-9-producing activity.^{7,8} IL-9 induces mast cell proliferation, goblet cell hyperplasia, BHR, and IL-13 production.^{9–12} Therefore, Th9 cells are recognized as potential targets for the treatment of bronchial asthma. Th9 cells can be generated from naive CD4⁺ T cells *in vitro* by priming with IL-4 and transforming growth factor-beta (TGF- β).⁸ In addition, differentiated Th2 cells subsequently produce IL-9 when they are cultured in the presence of TGF- β *in vitro*.⁸ Similar to Th2 cell-mediated models, mice transferred with Th9 cells

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develop asthma-like airway inflammation characterized by eosinophil infiltration and BHR in response to airway antigen challenge.¹³

In our previous study, we investigated the contribution of eosinophils to BHR using Th2- and Th9-transferred mice in which substantial accumulation of eosinophils in the lungs and BHR were induced by challenge with a specific antigen.¹⁴ An essential and dispensable role of eosinophils in Th2- and Th9-mediated BHR, respectively, was demonstrated by employing eosinophil-deficient mice.¹⁴

Glucocorticoids have been widely used for asthma therapy owing to their strong attenuating effects on airway eosinophilia by inducing eosinophil apoptosis.^{15,16} Regardless of the broad distribution of glucocorticoid receptors (GRs) in the airways, CD4⁺ T cells are primary targets of glucocorticoids. The number of CD4⁺ T cells expressing Th2 cytokines in the airways of asthmatic patients was shown to be reduced following glucocorticoid therapy in proportion to the extent of alleviation of their symptoms.^{17,18} In animal models, treatment with dexamethasone (Dex) attenuated the Th2 cell-mediated airway inflammation.^{19,20}

However, it has been demonstrated that 5–10% of asthmatic patients display severe and glucocorticoid-resistant phenotypes. In this regard, it has not been determined whether Dex treatment is effective for Th9 cell-mediated pathogenesis. Herein, by employing a murine adoptive transfer system for *in vitro*-differentiated Th cells, we investigated the effect of Dex on antigen-specific Th9 cell-induced airway inflammation, including BHR, in comparison to its effect on Th2 cell-mediated pathology.

Methods

Experimental animals

BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). DO11.10/RAG-2^{-/-} mice in a BALB/c background were maintained as described previously.^{6,14,21} All animal experiments were performed in accordance with guidelines approved by the Animal Use Committee at Tokyo Metropolitan Institute of Medical Science.

In vitro T cell differentiation

Th2 and Th9 cells were prepared as described previously.^{6,14,21} In brief, after depletion of erythrocytes, CD4⁺ T cells were isolated from the splenocytes of DO11.10/RAG-2^{-/-} mice using anti-mouse CD4 antibody (Ab)-conjugated magnetic beads and a magnetic cell sorting system (Miltenyi, Bergisch Gladbach, Germany). The cells were cultured in the presence of X-ray-irradiated syngeneic spleen cells as antigen-presenting cells and 0.3 μM ovalbumin (OVA) peptide (OVA323–339 peptide) in Dulbecco's modified Eagle medium-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 induced by adding 10 U/mL of recombinant human IL-2 (Shionogi, Osaka, Japan) and mouse IL-4 (PeproTech, NJ, USA), and 10 μg/mL anti-interferon-γ (IFN-γ) monoclonal Ab (R4-6A2, eBioscience, CA, USA). 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 monoclonal Ab. The cells were cultured for 7 days and then used for the adoptive transfer experiment.

To determine the integrity of polarization, the cells (1×10^5) were incubated with irradiated splenocytes (2×10^5) with or without 0.3 μM synthetic OVA323–339 peptide for 24 h. Then, the total RNA was extracted and subjected to reverse transcription using Super Script III reverse transcriptase (Thermo Fischer

Scientific, Waltham, MA, USA) and random primers (Toyobo, Osaka, Japan) for quantitative reverse transcription-polymerase chain reaction for *Il-4*, *Il-5*, *Il-9*, *Il-10*, *Il-13* and *Gra* using TaqMan probes (Thermo Fischer Scientific, Inc.) with a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) as described previously.²² To test the effect of Dex, the compound (10 nM) was added at the start of culture.

Concentrations of cytokines were measured by ELISA (eBiosciences and BioLegend, CA, USA), according to the manufacturer's instructions. Detection limits of IL-4 and IL-9 were 20, and 50 pg/ml, respectively.

Cell transfer and antigen challenge

The BALB/c mice were intravenously injected with cells (1×10^7) suspended in phosphate-buffered saline (PBS). Twenty-four hours 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, PA, USA) under isoflurane anesthesia as described previously.¹⁴ To evaluate the effect of Dex, the mice were subcutaneously injected with either Dex (5 mg/kg) or PBS twice, at 1 h before and at 24 h after the OVA challenge.

Bronchoalveolar lavage fluid (BALF) analysis

Seventy-two hours after the antigen challenge, bronchoalveolar lavage was performed by introducing 3×0.5 mL PBS into the lungs via a tracheal cannula. The number of leukocytes in the BALF was counted using a hemocytometer, and differential cell counts based on morphological 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 the BALF was determined by flow cytometry upon staining with anti-CD4-APC-eFluor780 (eBioscience) and anti-KJ1-26-PE (BioLegend).

Measurement of BHR

Seventy-two hours after the antigen challenge, the 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 the progressive change in respiratory system resistance (Rrs) following inhalation of increasing doses of aerosolized methacholine (MCh; Nacalai tesque, Kyoto, Japan) through an inline nebulizer.

Statistical analysis

The results are presented as the 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.

Results

Cytokine expression of differentiated T cells *in vitro*

To evaluate the phenotypes of *in vitro*-differentiated Th2 and Th9 cells, their cytokine mRNA expression profiles, and concentration of cytokines in the culture supernatant were examined. Th2

cells expressed *Il-4*, *Il-5*, *Il-13* and *Il-10*, and a small amount of *Il-9* following OVA stimulation (Fig. 1A–E). In contrast, the cells grown under the Th9 conditions expressed substantial amounts of *Il-9* and *Il-10*, whereas their *Il-4*, *Il-5*, and *Il-13* expression levels were significantly lower than those of Th2 cells. There were no detectable amounts of cytokines expressed in Th2 and Th9 cells without antigen stimulation. Furthermore, the preferential production of IL-4 by Th2 cells (Supplementary Fig. 1A) and IL-9 by Th9 cells (Supplementary Fig. 1B) were detected also at the protein levels, suggesting that the *in vitro* culture conditions were appropriate for the development of typical Th2 and Th9 cells.

Dex inhibited Th2- but not Th9-mediated airway inflammation

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 the BALF of both groups of antigen-challenged mice (Fig. 2A, D). The bronchial responsiveness to inhaled MCh was similarly augmented in the Th2- and Th9-transferred mice by OVA challenge (Fig. 2C, F). These results are consistent with our previous reports.¹⁴ We next investigated the effect of Dex on antigen-induced bronchial cellular infiltration and BHR. The treatment with Dex significantly inhibited the infiltration of eosinophils and lymphocytes in Th2-transferred mice (Fig. 2A). The tendency toward the suppression of IL-4 production in the lungs by Dex was observed (Fig. 2B). Consistently, Dex significantly suppressed Th2 cell-mediated BHR (Fig. 2C).

In contrast to the Th2 cell-transferred mice, Dex treatment did not affect the antigen-induced accumulation of eosinophils, lymphocytes, and neutrophils in Th9 cell-transferred mice (Fig. 2D). The weak but not significant down-regulation of IL-9 production in the lungs by Dex was observed (Fig. 2E). Furthermore, Th9 cell-mediated BHR was not attenuated by Dex (Fig. 2F). These results suggest that Th9-mediated airway inflammation is resistant to steroid therapy.

Mechanisms of steroid-resistance in Th9 cells

Glucocorticoids exert their biological effects through binding to GRs. Therefore, the expression of *Gra* was examined in Th2 and Th9 cells. Equivalent levels of *Gra* were expressed in Th2 and Th9 cells (Fig. 3A). Moreover, the antigen-induced expression of *Il-4*, *Il-5*, and *Il-13* in Th2 cells and of *Il-9* in Th9 cells was significantly suppressed by treatment with Dex (Fig. 3B–E), whereas Dex did not affect the survival of those cells (Supplementary Fig. 2). These results suggest that Dex has a suppressive effect, at least on the IL-9-producing activity of Th9 cells as with the IL-4-producing activity of Th2 cells, via its association with GR α .

Next, to investigate the effect of Dex on antigen-specific T cell migration, we examined the antigen-induced accumulation of transferred T cells in the lungs. As the non-specific distribution of infused T cells was observed in saline-challenged mice to some degree, significant infiltration of Th2 and Th9 cells was induced by OVA challenge. The infiltration of Th2 cells was significantly suppressed by Dex, whereas the effect on Th9 cell migration was weak (Fig. 4). These data suggest that Dex did not efficiently suppress Th9-mediated airway inflammation due to the lack of efficacy in preventing Th9 cell migration.

Discussion

The clinical importance of Th9 cells in bronchial asthma has been demonstrated based on the increased blood and sputum IL-9 levels in patients.^{23,24} In addition, several asthmatic patients show unresponsiveness to Dex in mediating the IL-9-producing activity of Th9-polarized T cells.²⁵ Our present findings that Dex did not affect Th9-mediated airway inflammation *in vivo* but suppressed IL-9 expression in Th9 cells *in vitro* are partly consistent and partly contradictory with those of previous investigations.

Several mechanisms leading to steroid-resistant asthma have been proposed, such as altered GR-binding affinity for

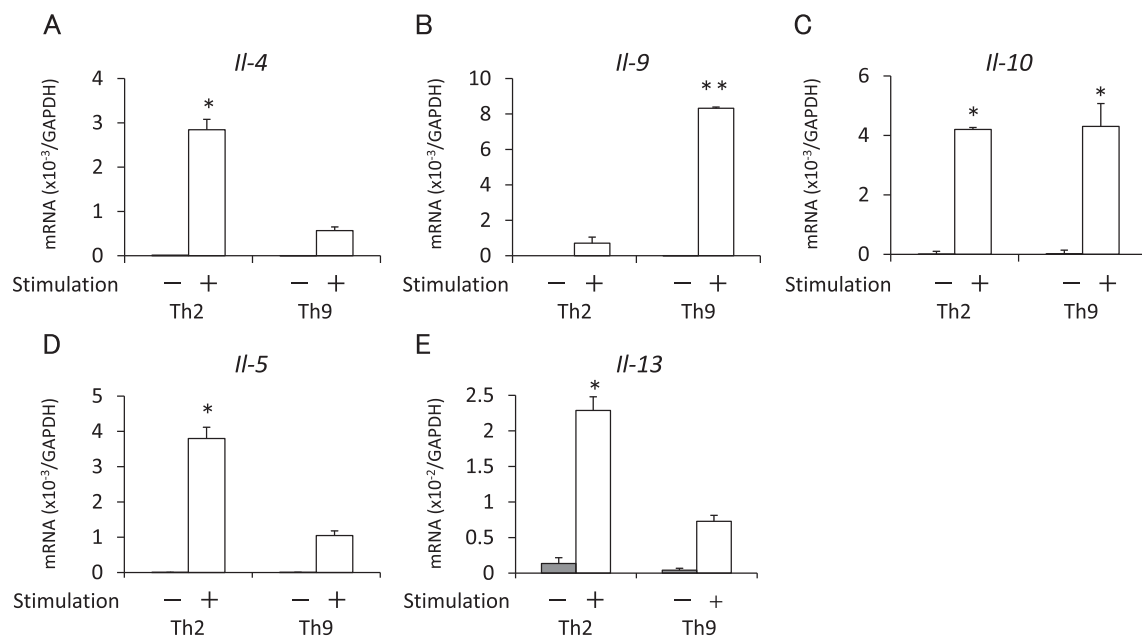


Fig. 1. Cytokine expression in Th2 and Th9 cells. *In vitro*-differentiated Th2 and Th9 cells were cultured with X-ray-irradiated splenocytes in the presence (Stimulation +) or absence (–) of OVA323–339 peptide. Twenty-four hours later, *Il-4*, *Il-9*, *Il-10*, *Il-5*, and *Il-13* mRNA expression levels were examined (A–E). Data are expressed as the mean $\pm$ SEM of triplicate data normalized with GAPDH expression. Essentially the same results were obtained in three independent experiments using distinct mice. * $p < 0.05$, ** $p < 0.01$, compared with the absence of OVA323–339 peptide.

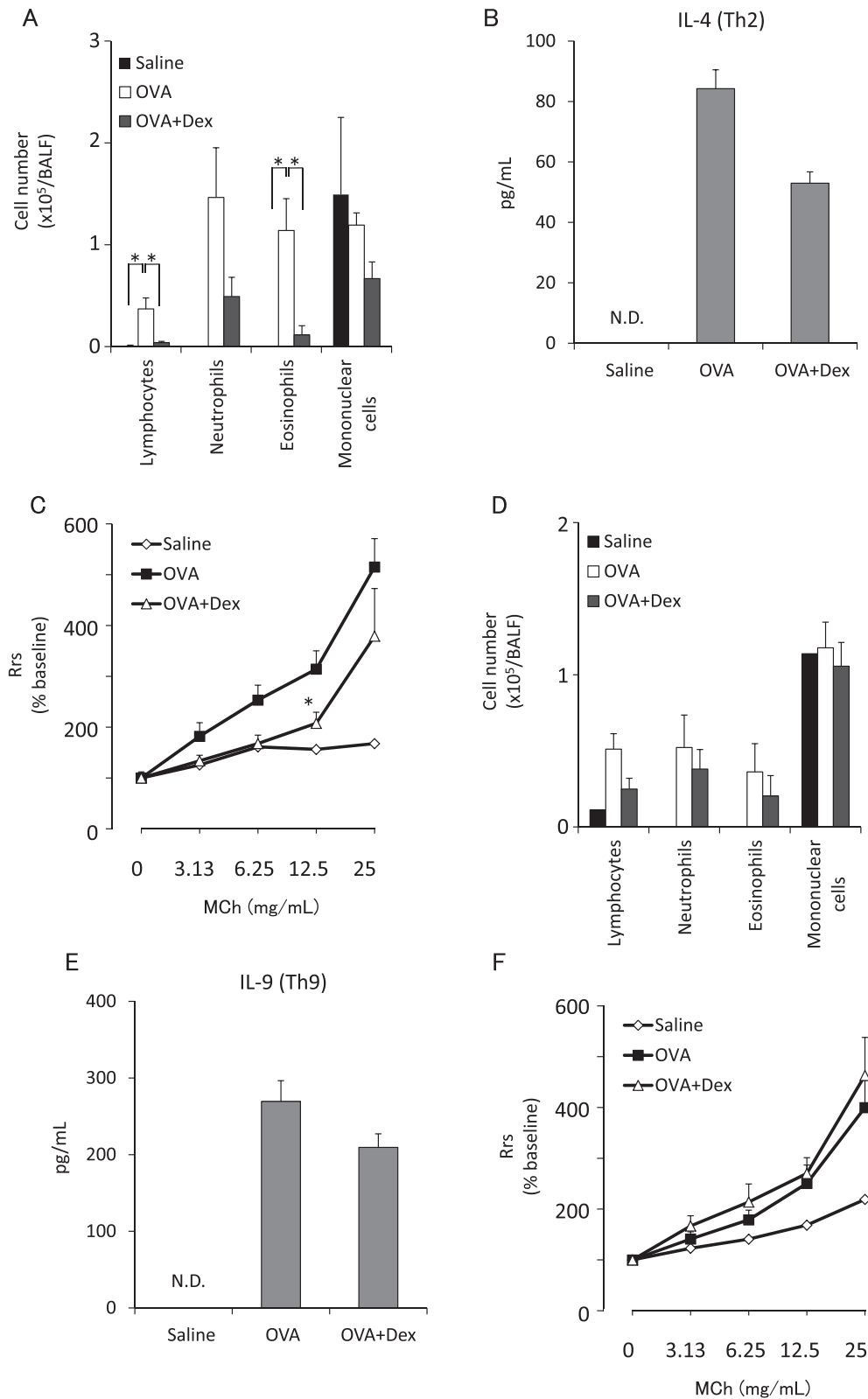


Fig. 2. Dexamethasone (Dex) did not attenuate Th9 cell-mediated airway inflammation. Th2 (A–C) or Th9 (D–F) cell-transferred mice were challenged with ovalbumin (OVA) or saline. Mice were treated with either Dex (5 mg/kg) or phosphate-buffered saline (PBS) as a control twice, at 1 h before and at 24 h after the OVA challenge, by subcutaneous injection. Seventy-two hours after the challenge, the numbers of inflammatory cells recovered in the bronchoalveolar lavage fluid (BALF) were differentially compared (A, D), the concentrations of IL-4 and IL-9 in the BALF were measured by ELISA (B, E), and the bronchial responsiveness to inhaled MCh was assessed (C, F). Data are expressed as mean $\pm$ SEM of 4–7 animals. * $p < 0.05$, compared with OVA-challenged mice. N.D., not detectable.

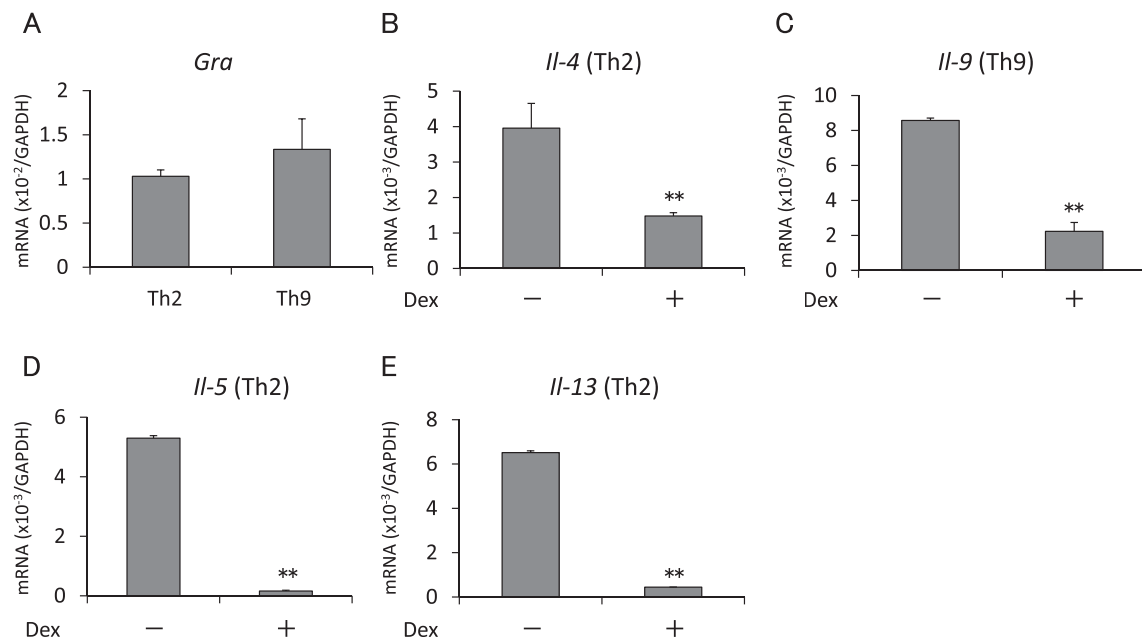


Fig. 3. Th9 cells were sensitive to dexamethasone (Dex) *in vitro*. Th2 and Th9 cells were stimulated with OVA323-339 peptide as described in Figure 1. *Gra* mRNA expression levels were examined in Th2 and Th9 cells without stimulation (A). Th2 (B, D, E) and Th9 (C) cells were stimulated in the presence and absence of 10 nM Dex. Twenty-four hours later, *Gra*, *Il-4*, *Il-9*, *Il-5* and *Il-13* mRNA expression levels were examined. ***p* < 0.01, compared with Dex-untreated control.

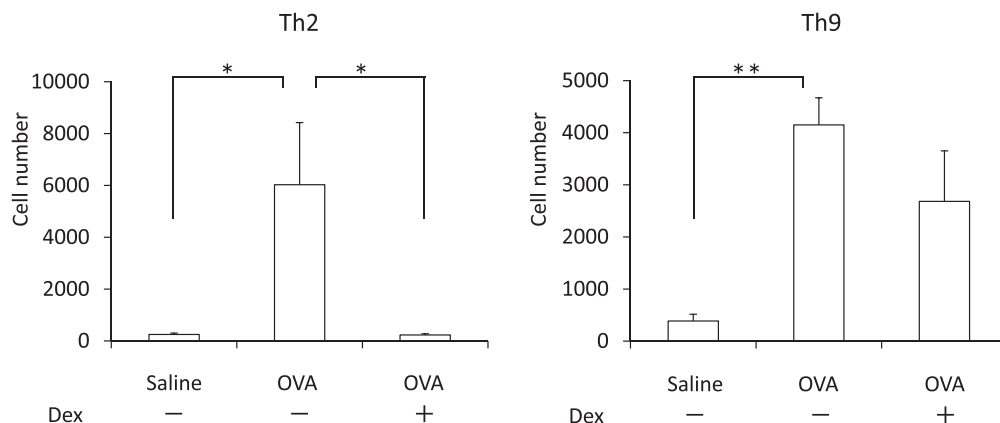


Fig. 4. The effect of dexamethasone (Dex) on airway infiltration of Th2 and Th9 cells. Ovalbumin (OVA) challenge and Dex treatment was performed in Th2 or Th9 cell-transferred mice as described in Figure 2. Seventy-two hours after the challenge, infused T cells recovered in the BALF were determined by flow cytometry. Data are expressed as mean $\pm$ SEM of 4–6 animals. **p* < 0.05, ***p* < 0.01, compared with Dex-untreated OVA-challenged mice.

glucocorticoids and GR numbers.^{26–28} In the present study, the murine Th9 cells expressed *Gra* to the same extent as Th2 cells; consequently, the Th9 cytokine response was sensitive to Dex at least *in vitro*. Human T cells express a dominant-negative GR isoform, GR β , that leads to a lack of response to glucocorticoids²⁹; however, murine cells do not express GR β .³⁰ The difference in the expression of GR isoforms might reflect the opposite effects of Dex on the IL-9 production by Th9 cells between mice and asthmatic patients.²⁴

Although antigen-induced IL-9 production in the lungs of Th9 cell-transferred mice was only partially inhibited by Dex *in vivo*, we previously demonstrated that Th9-mediated BHR was not dependent on IL-9 produced by Th9 cells.¹⁴ Therefore, a Th9-derived unknown mediator except for IL-9 might play a role in the development of steroid-resistant BHR in Th9 cell-transferred mice. It has

been described that innate lymphoid cells 2 (ILC2), which potentially contribute to eosinophilic airway inflammation, are relatively resistant to corticosteroid.³¹ Since IL-9 has been shown to promote the survival of ILC2, ILC2 might play a role in the steroid-resistance of Th9 cell-mediated airway inflammation.

Dex suppressed the infiltration of infused Th2 cells but not Th9 cells, suggesting that the lack of an effect on Th9 cell migration played a role in the unresponsiveness of Th9 cell-mediated airway inflammation to Dex. Although the mechanisms of the steroid-resistance of Th9 cell migration are unknown, Kara *et al.*³² reported that Th9 cells express functional CCR3, CCR6, and CXCR3, while Th2 cells preferentially express CCR3, and CCR4. Corticosteroids have been shown to upregulate the expression of IP-10, a ligand for CXCR3, in the asthmatic airway mucosa.^{33,34} Therefore, Dex might facilitate the infiltration of infused Th9 cells via the

upregulation of IP-10. Since the proportion of Th9 cells in total lymphocytes recovered in the BALF was relatively small, the upregulation of Th9 migration by Dex might not been detectable in the effect on lymphocyte migration. Further studies will be required to elucidate the mechanisms of Th9-mediated steroid-resistant airway inflammation in view of the regulation of chemokine-related responses.

In contrast, we previously demonstrated that antigen provocation in Th2 cell-transferred mice induced the production of TARC, a ligand for CCR4 which is highly expressed on Th2 cells, in the lungs.⁶ Sekiya *et al.* reported that the expression of TARC in bronchial epithelial cells was almost completely inhibited by glucocorticoids.³⁵ Therefore, the effect of Dex on Th2 cell migration might be due, at least in part, to the inhibition of TARC expression.

In several studies, glucocorticoid resistance has been shown to be caused by the abolition of apoptosis and IL-5-mediated survival signal inhibition of eosinophils.¹⁵ However, we previously demonstrated that Th9 cell-mediated BHR was not dependent on eosinophils infiltration in the lungs.¹⁴ Taken together with our present findings that Th9- but not Th2-mediated airway inflammation was resistant to Dex, new insights into the mechanisms of steroid-resistant asthma was provided.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.alit.2017.07.001>.

Conflict of interest

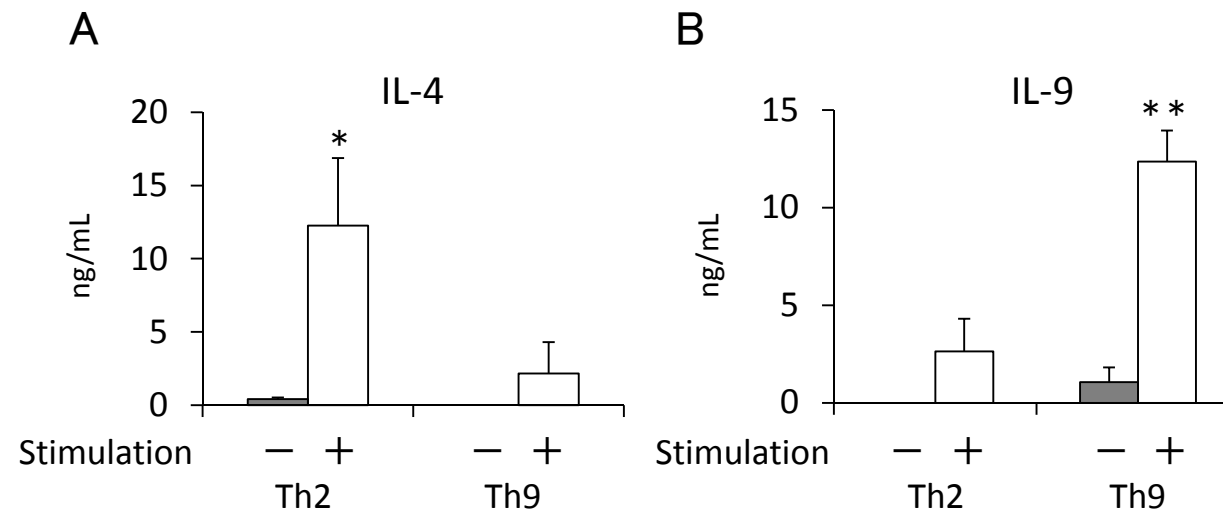
The authors have no conflict of interest to declare.

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.

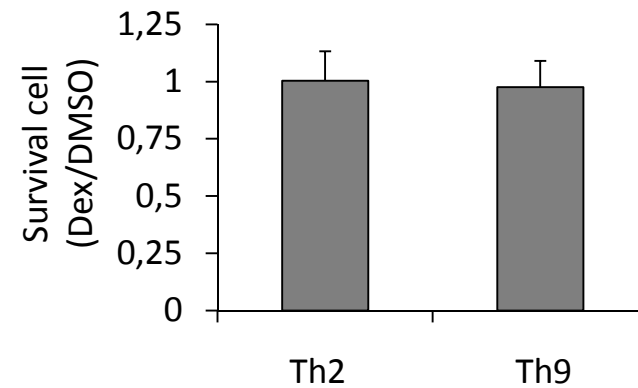
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Supplementary Fig. 1. Cytokine production in Th2 and Th9 cells.

In vitro-differentiated Th2 and Th9 cells were cultured with X-ray-irradiated splenocytes in the presence (Stimulation +) or absence (-) of OVA323-339 peptide. Twenty-four hours later, the concentrations of IL-4 and IL-9 in the culture supernatant were measured by ELISA. Data are expressed as the mean $\pm$ SEM of triplicate data normalized with GAPDH expression. *p < 0.05, **p < 0.01, compared with the absence of OVA323-339 peptide.



Supplementary Fig. 2. Dex did not affect the survival of Th2 and Th9 cells.

Th2 and Th9 cells were stimulated in the presence and absence of 10 nM Dex.

Twenty-four hours later, cell viabilities were measured.