

# Dual Role of Interleukin-23 in Epicutaneously-Sensitized Asthma in Mice

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## ABSTRACT

**Background:** Interleukin (IL)-23/Th17 axis plays an important role in the pathophysiology of asthma and eczema, however, there are some conflicting data about the effects of this system on allergic airway inflammation. In the present study, we aim to dissect the spatiotemporal differences in the roles of IL-23 in an epicutaneously-sensitized asthma model of mice.

**Methods:** C57BL/6 mice were sensitized to ovalbumin (OVA) by patch application on the skin, followed by airway exposure to aerosolized OVA. During sensitization and/or challenge phase, either a specific neutralizing antibody (Ab) against IL-23 or control IgG was injected intraperitoneally. On days 1 and 8 after the final OVA exposure, airway inflammation and responsiveness to methacholine, immunoglobulin levels in serum, and cytokine release from splenocytes were evaluated. Skin *Il23a* mRNA levels were evaluated with quantitative RT-PCR.

**Results:** Patch application time-dependently increased the expression of *Il23a* mRNA expression in the skin. Treatment with the anti-IL-23 Ab during sensitization phase alone significantly reduced the number of eosinophils in bronchoalveolar lavage fluids and peribronchial spaces after allergen challenge compared with treatment with control IgG. Anti-IL-23 Ab also reduced serum levels of OVA-specific IgG<sub>1</sub>. In contrast, treatment with the anti-IL-23 Ab during the challenge phase alone rather exacerbated airway hyperresponsiveness to methacholine with little effects on airway eosinophilia or serum IgG<sub>1</sub> levels.

**Conclusions:** IL-23 expressed in the skin during the sensitization phase plays an essential role in the development of allergic phenotypes, whereas IL-23 in the airways during the challenge phase suppresses airway hyperresponsiveness.

## KEY WORDS

airway hyperresponsiveness, atopic march, eosinophils, IgG<sub>1</sub>, skin

## ABBREVIATIONS

Ab, antibody; AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; IFN- $\gamma$ , interferon gamma; Ig, immunoglobulin; IL, interleukin; OVA, ovalbumin; PAS, periodic acid-Schiff; PBS, phosphate buffered saline; SEM, standard errors; Th, helper T.

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Conflict of interest: YS and SK received salary from MSD Endowed Program for Allergy Research. TB received research funding from GlaxoSmithKline. KA received honoraria from Glaxo-

SmithKline and research funding from GlaxoSmithKline, Astellas. The rest of the authors have no conflict of interest.

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Received 27 September 2013. Accepted for publication 29 January 2014.

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## INTRODUCTION

Asthma is a chronic inflammatory disease in the airways characterized by eosinophilic inflammation, bronchial hyperresponsiveness, and remodeling such as goblet cell hyperplasia in the airway epithelium. Most patients, especially those who developed asthma during the childhood, exhibit sensitization to allergens such as house dust mite, animal dander, and fungi. Importantly, the same allergen(s), especially house dust mite, plays an important role in the pathogenesis of other allergic diseases such as allergic rhinitis and eczema, and therefore, it is not surprising that a substantial number of patients suffer from more than one allergic diseases. In the cases with so-called 'atopic march', eczema often precedes the development of allergic symptoms in the upper and lower airways, suggesting the importance of epicutaneous sensitization in these patients.<sup>1-5</sup> This hypothesis is further supported by the discovery that loss-of-function mutations in the gene of filaggrin, a molecule indispensable for normal cutaneous barrier function, increase the risk of both eczema and asthma.<sup>2,6-9</sup> It is, therefore, important to study the role of the skin as an interface and initial immune response to exogenous allergen in the process of atopic march.

Recent studies have clarified that IL-23, a cytokine that promotes differentiation and survival of Th17 cells and production of IL-17A, IL-17F, and IL-22,<sup>10,11</sup> enhances Th2-type immune reactions<sup>12</sup> and is associated with pathogenesis and severity of asthma.<sup>13-16</sup> In addition to the evidences obtained from the studies using intraperitoneally-sensitized models, clinical and basic research has elucidated that IL-23/Th17 pathway is more crucial in the immune responses to allergens in the skin. The number of Th17 cells is increased in the skin and in the peripheral blood of patients with atopic dermatitis, associating with its severity.<sup>17</sup> Epicutaneous, but not intraperitoneal immunization of mice to ovalbumin (OVA) expands the number of IL-17A-producing T cells in draining lymph nodes and spleen, and increases serum levels of IL-17A.<sup>18</sup>

There is, however, some controversy about the role of IL-23/Th17 axis in the pathophysiology of allergic airway diseases. Exogenous IL-17A, given to mice during established asthma inflammation, reduces eosinophil recruitment into the lungs and airway hyperresponsiveness.<sup>19</sup> Other researchers also reported that exogenous IL-23 treatment reduces airway hyperresponsiveness and inflammation in asthmatic *Tlr6*<sup>-/-</sup> mice.<sup>20</sup> These discrepancies among the studies may be due to the differences of IL-23 and IL-17 expression in regard to the timing and/or the location. In the present study, we separately blocked IL-23 activity during the sensitization and the exposure phase using an IL-23-specific neutralizing antibody, in

order to examine the spatiotemporal differences in the roles of IL-23 in epicutaneously-sensitized asthma.

## METHODS

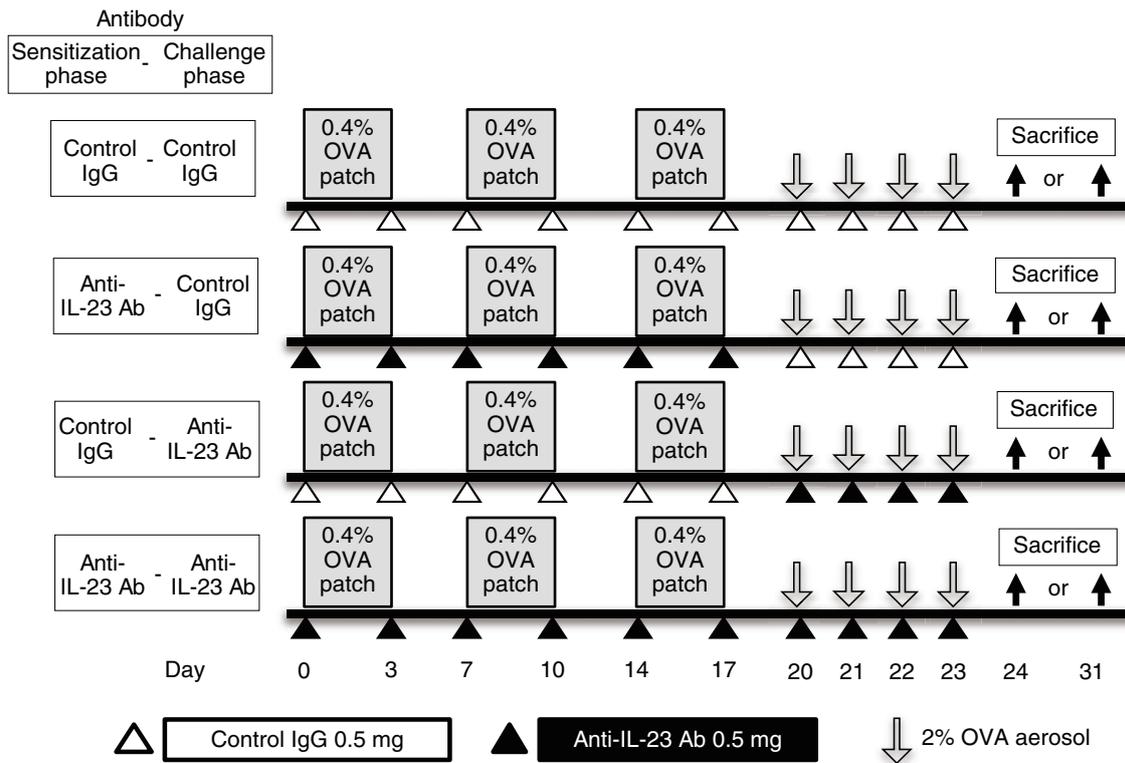
### ANIMALS AND PROTOCOLS FOR EPICUTANEOUSLY-SENSITIZED ASTHMA MODEL

Specific pathogen-free, 6-week-old, male C57BL/6J mice, weighing between 25 and 30 g, purchased from Charles River Laboratories, Kanagawa, Japan, were used in this study. Epicutaneously-sensitized asthma model was prepared as previously reported.<sup>21</sup> Briefly, 40  $\mu$ l phosphate buffered saline (PBS) containing 160  $\mu$ g ovalbumin (Sigma, St. Louis, MO, USA) was placed on the filter paper in a Finn chamber (Smart-Practice<sup>®</sup>, Phoenix, AZ, USA) and applied to the shaved skin on the back of mice on days 1-3, 8-10, and 15-17 (Fig. 1). These mice were then exposed to aerosolized allergen [2% (w/v) OVA diluted in PBS] for 20 min in a dedicated chamber on days 20-23. During the sensitization and/or the challenge phase, anti-IL-23 Ab (CNTO 6163, 0.5 mg/mice, provided by Johnson & Johnson, New Brunswick, NJ, USA) or control IgG Ab (CNTO 1322, 0.5 mg/mice, provided by Johnson & Johnson) was injected intraperitoneally as shown in the Figure 1.

This study followed the Helsinki Convention standards for the use and care of animals. All the protocols of the animal experiments used in this study were approved by the Laboratory Animal Care and Use Committee of Keio University School of Medicine.

### MEASUREMENT OF AIRWAY RESPONSIVENESS

The airway responsiveness to methacholine (Sigma) was measured by using flexiVent system (SCIREQ, Montreal, Canada) at 1 and 8 days after the last challenge of aerosolized OVA. The animals anesthetized with ketamine and xylazine were quasi-sinusoidally ventilated at 180 breaths/min, with a computer-controlled, small animal ventilator, with a tidal volume of 10 ml/kg, against an artificial positive end-expiratory pressure of 3 cm H<sub>2</sub>O. Resistance of respiratory system was measured, using a 3-Hz sinusoidal piston volume movement of 10 ml/kg. The pressure-volume and flow data were fit to a single compartment model to derive the measurements of airway resistance. Freshly prepared methacholine in cold PBS was delivered through an inline nebulizer, in doses of 0, 6.25, 12.5, 25, 50 and 100 mg/ml. The direct delivery of methacholine to the lung was timed with inspiration. After each methacholine challenge, the airway resistance was measured every 15 s during tidal breathing, and the 3<sup>rd</sup> or 4<sup>th</sup> measurement whichever was higher was used as the value of bronchoconstrictor response to each individual methacholine concentration.



**Fig. 1** Experimental protocol for epicutaneously-sensitized asthma model. Mice were divided into 4 groups in terms of the antibodies (control IgG or anti-IL-23 antibody) and the timing of administration (sensitization or challenge phase). On 1 or 8 days after the last ovalbumin challenge, airway hyperresponsiveness was measured and then mice were sacrificed. OVA, ovalbumin; Ab, antibody.

**ASSAY OF SERUM IMMUNOGLOBULIN LEVELS**

Mice were sacrificed using overdose pentobarbital after the measurement of airway responsiveness, and whole blood was collected from the inferior vena cava. Serum samples were tested by sandwich ELISAs for total IgE and OVA-specific IgG<sub>1</sub>. Total IgE levels were measured using plates coated with purified rat anti-mouse IgE capture Ab (BD Biosciences, San Jose, CA, USA). OVA-specific IgG<sub>1</sub> was measured by direct ELISA using plates coated with OVA and a biotin rat anti-mouse IgG<sub>1</sub> (BD Biosciences) as detection Ab. The concentration of each immunoglobulin was given in absorbance (OD 405 nm) values.

**BRONCHOALVEOLAR LAVAGE FLUID**

The trachea was intubated and gently flushed with 1.4 ml of cold PBS including 0.6 mM ethylenediamine tetraacetic acid (EDTA). Total number of cells in BALF was determined with hemocytometer after hemolysis, and differential cell counts were determined in cytopsin preparations stained with Diff-Quick (Symex, Kobe, Japan).

**LUNG HISTOLOGY**

The chest was opened, and pulmonary circulation was thoroughly flushed with PBS using a peristaltic

pump (flow rate 5 ml/min) through a catheter inserted in the pulmonary artery. The left lung lobes were removed and fixed in 4% (w/v) neutralized buffered paraformaldehyde (pH 7.4). Lung tissues were paraffin embedded, and the sliced sections were stained with hematoxylin & eosin or periodic acid Schiff (PAS)-alcian blue for histological analyses. A semi-quantitative scoring system was used to grade the degree of eosinophil accumulation and mucus production in the airway epithelium. In the tissues stained with hematoxylin & eosin, randomly-selected 5 bronchi were graded from 0 (no eosinophils) to 4 (abundant eosinophilic infiltration) in a blinded manner, and scores were summed (0-20). In the tissues stained with PAS-alcian blue, mucus production in the airway epithelium was also semi-quantified. We evaluated the all bronchi in the tissues and graded from 0-4 (proportion of PAS-alcian blue positive epithelial cells in each bronchus: 0%: 0, 1-25%: 1, 26-50%: 2, 51-75%: 3, 76-100%: 4), and a mean score was used as mucus score.

**ISOLATION OF SPLEEN CELLS FOR *in vitro* STIMULATION WITH ALLERGEN**

Spleen was minced and cells were isolated using cell strainers (40 μm). Spleen cells were cultured *in vitro*

in RPMI1640 with 10% FBS in the absence or presence of OVA (100 µg/mL) for 3 days. The culture supernatants were measured for Th1, Th2 and Th17 cytokines with ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

### QUANTITATIVE RT-PCR

Skin tissues were biopsied at 1, 3, and 6 h after OVA patch application. The tissue samples were homogenized, RNA was extracted using RNeasy mini kit (QIAGEN, Hilden, Germany), and cDNA was generated with SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The expression levels of *Il12a*, *Il12b*, *Il23a*, *Gapdh* mRNAs were measured by real-time quantitative PCR using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and an ABI 7500 real-time PCR system (Applied Biosystems). Data were normalized to the expression levels of *Gapdh* gene.

### STATISTICAL ANALYSIS

All data are presented as means and standard errors (SEM). Data were analyzed with GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). The Student's t-test was used to determine statistical significance between two groups. One-way ANOVA, followed by Dunnett's test as a *post hoc* test, was used to compare the three groups treated with anti-IL-23 Ab and the group treated with control IgG alone. Two-way ANOVA were used for the analyses of airway responsiveness and gene expression in the skin. All reported *P* values were based on two-sided tests.

## RESULTS

### IL-23 GENE EXPRESSION IN THE SKIN

We hypothesized that the skin was one of the sources of IL-23 production in response to allergen or mechanical stress during epicutaneous sensitization. PBS or OVA patch was placed on the shaved skin of mice for up to 6 hours, and then the skin was biopsied. There was no change in the levels of *Il17a*, *Il17f*, *Il12a* (the gene for IL-12p35), or *Il12b* (the gene for IL-12p40 which is the common component of IL-12 and IL-23). In contrast, the mRNA levels of IL-23p19 (*Il23a*) were significantly increased than the levels in the naïve skin at 6 h after the patch application (Fig. 2). There was no difference whether the patch contains OVA or not, suggesting the role of mechanical stress on the skin in the induction of IL-23 expression.

### ANTI-IL-23 Ab REDUCES THE SERUM LEVELS OF OVA-SPECIFIC IgG<sub>1</sub>

The serum levels of total IgE and OVA-specific IgG<sub>1</sub> in OVA-sensitized and challenged mice were significantly elevated than those of naïve mice (Fig. 3). Treatment with anti-IL-23 Ab during both sensitization and challenge phase decreased the levels of

OVA-specific IgG<sub>1</sub> compared to those treated with control IgG alone (Fig. 3b, *p* < 0.01). Treatment with anti-IL-23 Ab during sensitization phase alone also showed a trend to decrease the levels of IgG<sub>1</sub>, but did not reach the statistical significance (Fig. 3b, *P* = 0.08). We could not find any significant difference in the serum levels of total IgE among the 4 groups treated with anti-IL-23 Ab and/or control IgG, due to large inter-animal variability (Fig. 3a). OVA-specific IgE was not detectable in this model as we previously reported.<sup>21</sup>

### ANTI-IL-23 Ab DURING SENSITIZATION PHASE REDUCES AIRWAY EOSINOPHILIA

On the next day after the last allergen challenge, there was no difference in the total and differential counts of inflammatory cells in BALF among 4 groups treated with anti-IL-23 Ab and/or control IgG (Fig. 4a). In contrast, the number of eosinophils on day 8 after allergen challenge was significantly reduced in the animals that received anti-IL-23 Ab treatment only in the sensitization phase (Fig. 4b). Anti-IL-23 Ab treatment during the challenge phase alone did not show any effects on BALF eosinophilia. There were few neutrophils in BALF on days 1 and 8 after allergen challenge, without any difference by the anti-IL-23 Ab treatment.

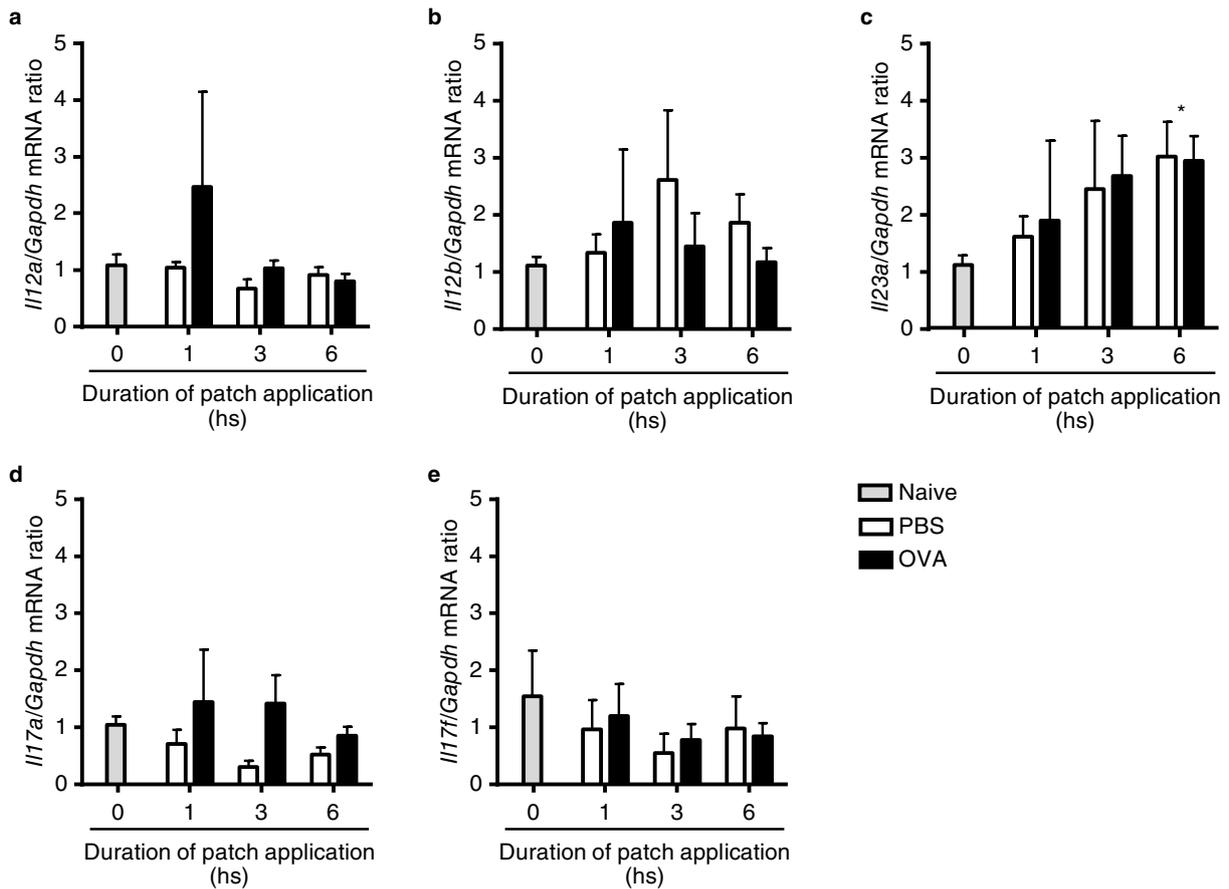
Histological examination of the lungs harvested 8 days after OVA exposure also demonstrated that anti-IL-23 Ab treatment during sensitization phase significantly reduced eosinophil accumulation in the peribronchial space (Fig. 5a). As for mucus score, we did not find any difference among the groups (Fig. 5b).

### ANTI-IL-23 Ab DURING CHALLENGE PHASE EXACERBATES AIRWAY HYPERRESPONSIVENESS

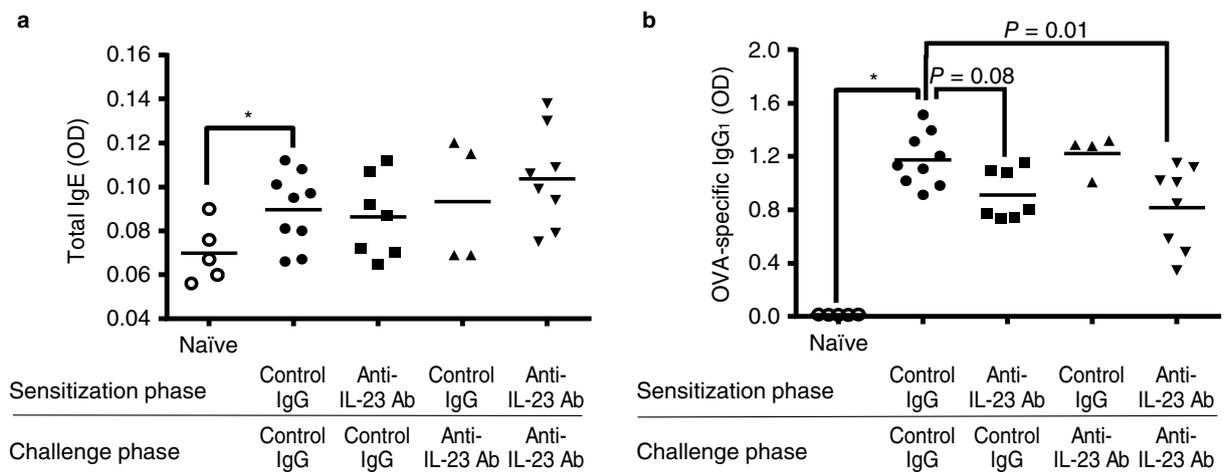
Enhanced airway hyperresponsiveness was observed both on days 1 and 8 in the mice sensitized and challenged with OVA (Fig. 6). Mice administrated with anti-IL-23 Ab during the challenge phase alone revealed a significant increase in the airway hyperresponsiveness on day 8 than those treated with control IgG Ab alone. Treatment with anti-IL-23 Ab during sensitization phase alone did not affect airway hyperresponsiveness.

### EFFECTS OF ANTI-IL-23 Ab ON THE CYTOKINE SYNTHESIS IN SPLEOCYTES

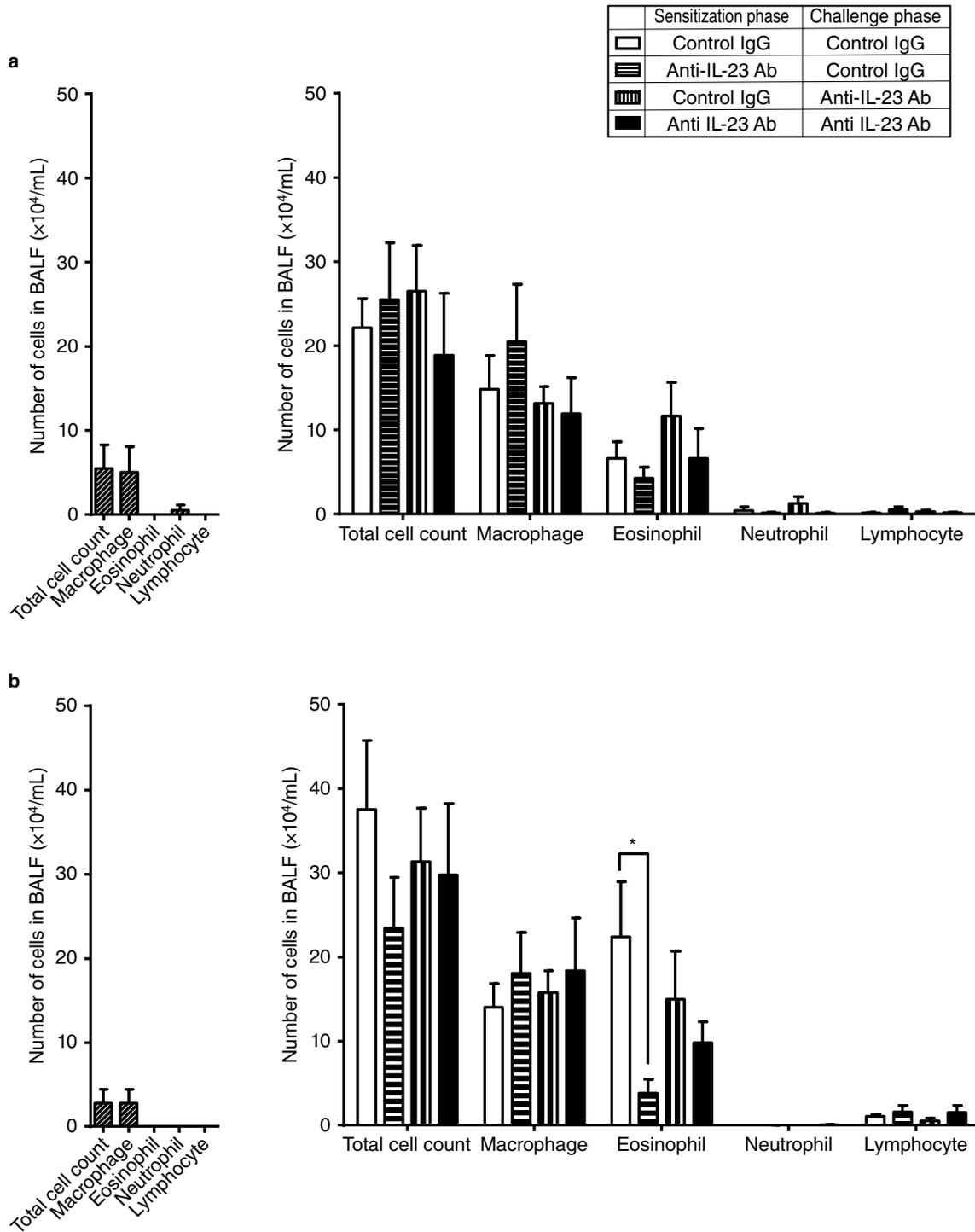
Spleen cells, isolated from epicuaneously-sensitized and challenged mice, showed a significant increase in the release of IL-13, IL-17A, IL-17F, and interferon gamma (IFN-γ) in response to the re-stimulation with OVA *in vitro*. Splenocytes from the animals treated with anti-IL-23 Ab during sensitization phase showed a trend to decrease the production of IL-13 and IL-17A, but did not reach statistical significance (Fig. 7).



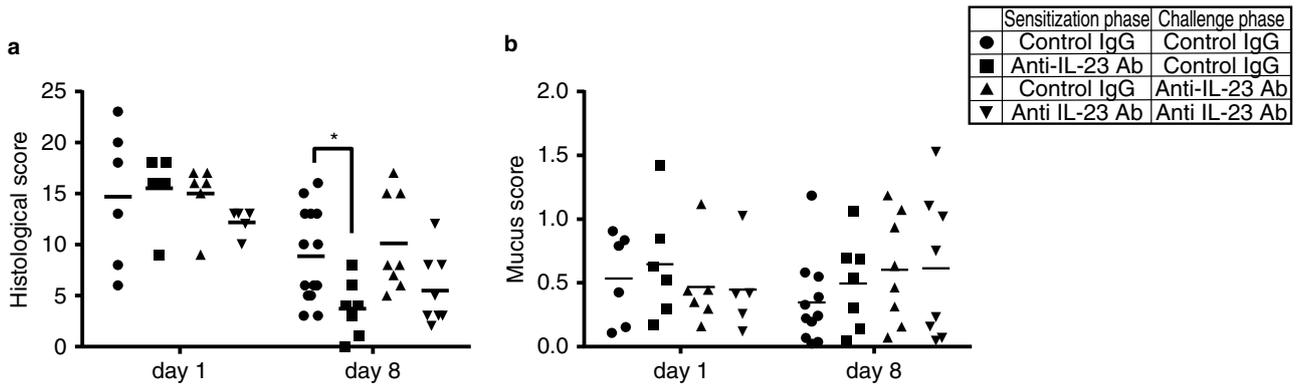
**Fig. 2** Expression of *Il12a* (a), *Il12b* (b), *Il23a* (c), *Il17a* (d), and *Il17f* (e) mRNAs in the skin applied with patch containing phosphate buffered saline (PBS: open columns) or ovalbumin (OVA: closed columns) for 0, 1, 3, and 6 hs. The levels of cytokine mRNAs determined by quantitative RT-PCR were normalized with the expression of *Gapdh* mRNA. The expression levels at 0 h was defined as 1.0. Mean + SEM,  $n = 3-10$  for each group. \* $P < 0.05$  compared to the levels at 0 h (naïve mice).



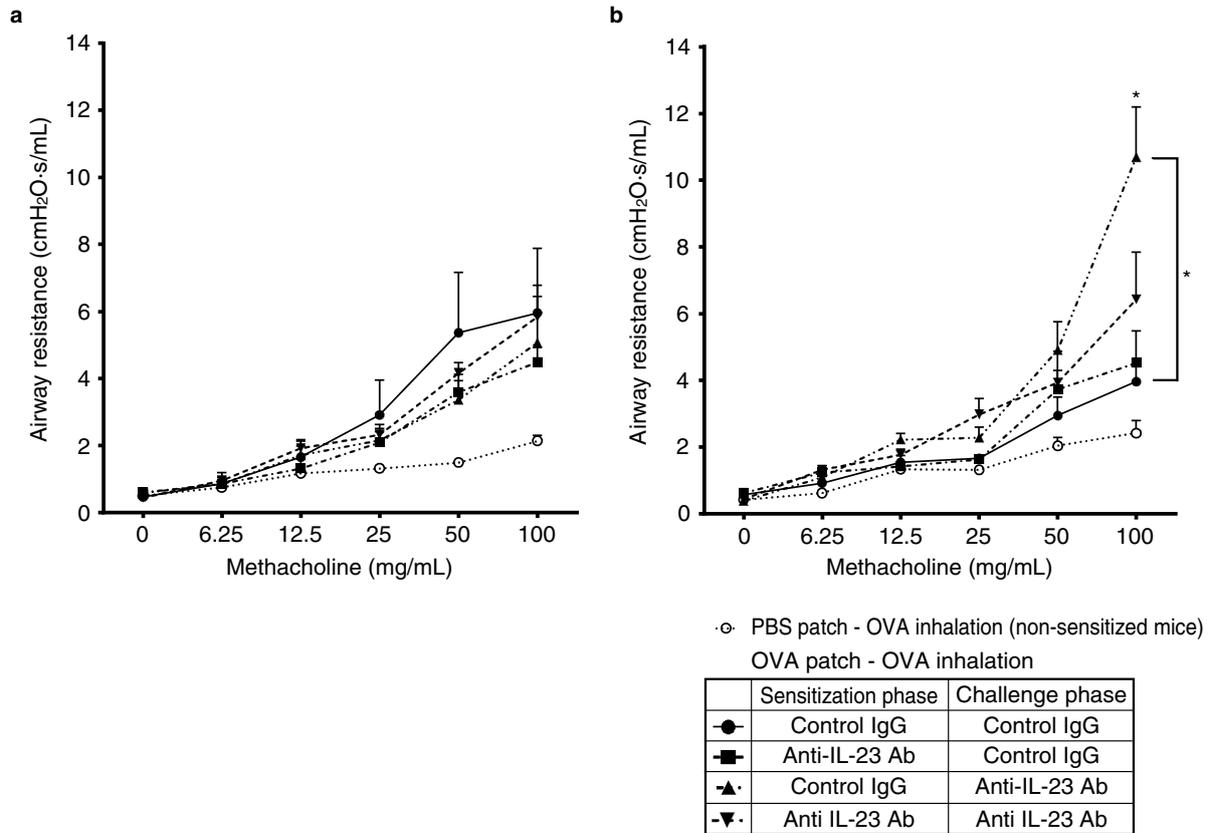
**Fig. 3** Serum levels of total IgE (a) and OVA-specific IgG<sub>1</sub> (b) in epicutaneously-sensitized mice on day 8 after the last OVA exposure. Bars represent the mean values. \* $P < 0.05$  compared to naïve animals.



**Fig. 4** Total and differential cell counts in bronchoalveolar lavage fluid (BALF) on day 1 (a) or day 8 (b) after the last ovalbumin (OVA) exposure. Left panels show the mice which received phosphate buffered saline (PBS) patch and then exposed to OVA aerosols ( $n = 3$ ). Right panels demonstrate the mice treated with OVA patch and OVA inhalation ( $n = 5-17$ ). Mean + SEM. \* $P < 0.05$  compared to the animals treated with control IgG during both the sensitization phase and the challenge phase.



**Fig. 5** Histological score of eosinophil accumulation in peribronchial spaces (a) and mucus production in the airway epithelium (b) on day 1 or 8 after the last ovalbumin (OVA) exposure. Bars represent the mean values. \**P* < 0.05 compared to the animals treated with control IgG during both the sensitization phase and the challenge phase.

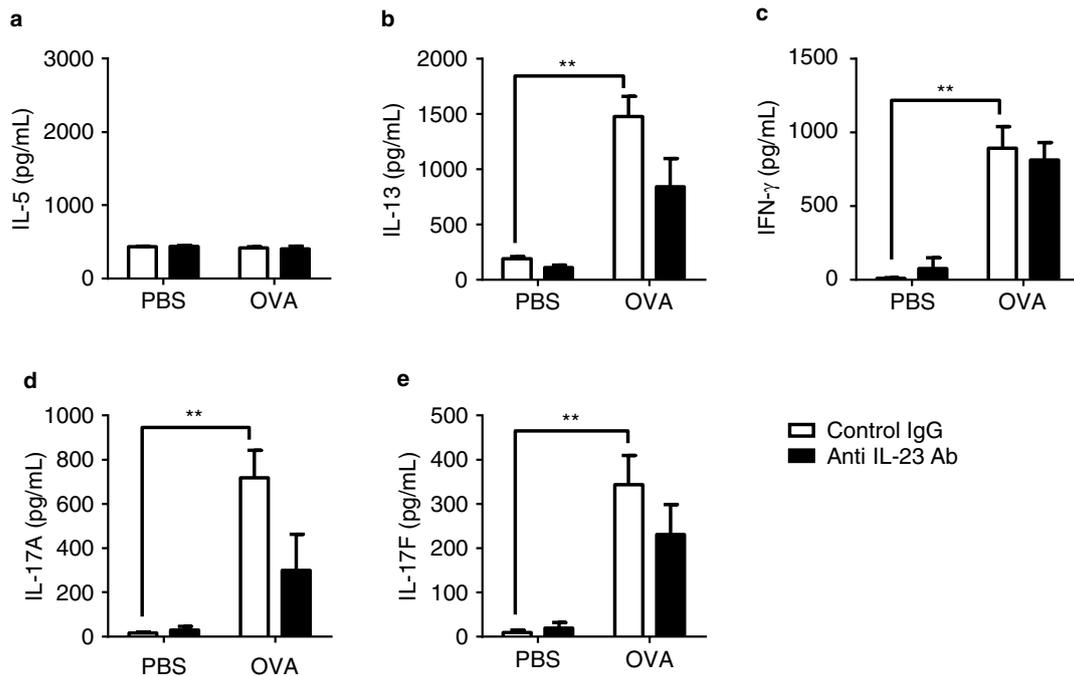


**Fig. 6** Airway hyperresponsiveness to methacholine (6.25-100 mg/mL) on day 1 (a) or day 8 (b) after the last ovalbumin (OVA) exposure. Mean  $\pm$  SEM, *n* = 3 for mice sensitized with PBS and *n* = 5-6 for those sensitized with OVA. \**P* < 0.05, compared to the animals treated with control IgG during both the sensitization phase and the challenge phase.

**DISCUSSION**

Patch application used in the present study for the epicutaneous sensitization, as tape stripping protocols used in the previous reports,<sup>18,22</sup> upregulated the expression of IL-23 in the skin regardless of the pres-

ence of OVA, suggesting the role of mechanical stress. It has been demonstrated that mechanical stress on epidermis induces the release of adenosine 5'-triphosphate,<sup>23</sup> which can enhance IL-23 expression in dendritic cells.<sup>24</sup> The source of IL-23 in the skin is still unclear, although IL-23 signaling is



**Fig. 7** Production of IL-5 (a), IL-13 (b), IFN- $\gamma$  (c), IL-17A (d), and IL-17F (e) in spleen cells from epicutaneously-sensitized asthmatic mice treated with or without anti-IL-23 antibody. Splenocytes isolated on the next day after the last ovalbumin (OVA) exposure were re-stimulated *in vitro* with phosphate buffered saline (PBS) or OVA for 3 days. Mean + SEM,  $n = 3-5$  for each groups. \*\* $P < 0.01$  compared to the splenocytes re-stimulated with PBS.

enhanced in keratinocyte<sup>25</sup> and antigen presenting cells including Langerhans cells and dermal dendritic cells<sup>26-28</sup> in the psoriasis skin. The present study demonstrates that the blockade of IL-23 during this sensitization phase with a neutralizing antibody caused important changes in the immune responses of the host; 1) inhibition of antigen-specific IgG1 synthesis, and 2) mitigation of the persistent eosinophilic airway inflammation. On the other hand, the inhibition of IL-23 during the challenge phase of allergen to the airways rather exacerbated airway hyperresponsiveness.

There are several possibilities in the mechanism how the activation of IL-23/Th17 pathway enhances the allergen sensitization and the following airway inflammation. Vaccination against IL-23p40, the common subunit for IL-12 and IL-23, suppresses neutrophilic, but not eosinophilic inflammation in the airways of the epicutaneously-sensitized asthma model,<sup>16</sup> suggesting the role of IL-23-driven production of IL-17 that stimulates airway epithelial cells to release neutrophilic chemokines. In contrast, our model lacked neutrophilic inflammation even in the absence of IL-23 blockade, and moreover, the anti-inflammatory effects of anti-IL-23 antibody were observed only when it was administered during the sensitization, but not in the challenge phase. Therefore, there should be other mechanisms for IL-23 to enhance airway inflammation than the recruitments of

neutrophils. In fact, both IL-23p40 vaccine and anti-IL-23 Ab suppressed the levels of OVA-specific IgG1, suggesting that IL-23 blockade exhibits some mechanism(s) to dampen Th2 immune responses.

There are accumulating evidences indicating that IL-23 and its signaling is important for the induction of Th2 immune responses. Enforced expression of IL-23 in the murine airways significantly increases not only allergen-induced release of IL-17A, but also of IL-13 in the BALF with enhanced airway inflammation and hyperresponsiveness to acetylcholine.<sup>15</sup> Similarly, overexpression of IL-23 receptor in lymphocytes results in enhanced Th2 cytokine production,<sup>12</sup> which is further supported by the *in vitro* experiments showing the role of IL-23 in the differentiation of Th2 cell.<sup>15</sup> These and other reports are consistent with our observation that the inhibition of IL-23 during the sensitization phase was associated with a trend of decreased production of systemic Th2/Th17 cytokines.<sup>12,20</sup>

IL-17A can also act as a negative regulator of eosinophil recruitment and hyperresponsiveness in the airways, when administrated in the established asthmatic inflammation.<sup>19</sup> These activities of IL-17A to reduce pulmonary eosinophilia may be associated with suppressed expression of chemokines such as CCL11/eotaxin.<sup>19</sup> In the present study, we found that administration of anti-IL-23 Ab during the antigen

challenge phase alone enhances airway hyperresponsiveness. In addition, the effects of anti-IL-23 Ab on the reduction of pulmonary eosinophilia were diminished when the antibody was administered during both sensitization and challenge phase.

Interestingly, we found some discrepancy between eosinophilic airway inflammation and bronchial hyperresponsiveness after the treatment with anti-IL-23 antibody during either sensitization or challenge phase. Such dissociation between airway inflammation and hyperresponsiveness has been reported in other models of asthma,<sup>29-31</sup> however, its exact mechanisms are yet to be determined.

In conclusion, the blockade of IL-23 is beneficial when it is applied during the sensitization phase. In the clinical practice, however, temporal separation of sensitization and challenge phases is impossible. Therefore, specific inhibition of IL-23 expression or signaling in the skin would be an appropriate approach to modulate epicutaneous sensitization. Because large molecules such as antibodies cannot be locally delivered to the skin due to its barrier function, the development of low molecular weight compounds that block IL-23 signaling is expected to control epicutaneous sensitization.

## ACKNOWLEDGEMENTS

We thank Miyuki Yamamoto and Yuko Hashimoto for their excellent technical support.

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