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Distinct effects of endogenous interleukin-23 on eosinophilic airway inflammation in response to different antigens

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Abbreviations:

BALF, bronchoalveolar lavage fluid;
EDTA, ethylenediaminetetraacetic acid;
ELISA, enzyme-linked immunosorbent
assay; GAPDH, glyceraldehyde-3-phosphate
dehydrogenase; HDM, house dust mite; IFN- γ ,
interferon gamma; Ig, immunoglobulin;
IL, interleukin; OVA, ovalbumin;
PBS, phosphate buffered saline; (RT-)
PCR, (reverse transcription) polymerase
chain reaction; SEM, standard error of the
mean; Th, helper T; TLR, toll-like receptor

ABSTRACT

Background: The role of interleukin (IL)-23 in asthma pathophysiology is still controversial. We examined its role in allergic airway inflammation in response to two distinct antigens using IL-23-deficient mice.

Methods: Allergic airway inflammation was evaluated in wild-type and *IL-23p19*^{-/-} mice. Mice were sensitized to ovalbumin (OVA) or house dust mite (HDM) by intraperitoneal injection of antigen and their airways were then exposed to the same antigen. Levels of antigen-specific immunoglobulins in serum as well as cytokines in bronchoalveolar or peritoneal lavage fluid and lung tissue were determined by enzyme-linked immunosorbent assay and/or quantitative polymerase chain reaction.

Results: Deficiency of *IL-23p19* decreased eosinophils and Th2 cytokines in bronchoalveolar lavage fluid (BALF) of OVA-treated mice, while it increased BALF eosinophils of HDM-treated mice. Peritoneal injection of OVA with alum, but not of HDM, induced local synthesis of IL-6, IL-10, and IL-23. Systemic production of antigen-specific IgG₁ was partially dependent on IL-23. In contrast, airway exposure to HDM, but not to OVA, induced *IL-23p19* mRNA expression in the lungs. In *IL-23p19*-deficient mice, HDM-exposed lungs did not exhibit the induction of *IL-17A*, which negatively regulates eosinophilic inflammation.

Conclusions: Different antigens induced IL-23 at different part of the body in our similar asthma models. Endogenous IL-23 production at the site of antigen sensitization facilitates type-2 immune responses, whereas IL-23 production and subsequent IL-17A synthesis in the airways suppresses allergic inflammation.

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Introduction

Bronchial asthma is a chronic inflammatory disease of the airways characterized by eosinophilic infiltrates, mucus hypersecretion, airway remodeling, and bronchial hyperresponsiveness.¹ Allergic airway inflammation is primarily driven by allergen-specific CD4⁺ T cells. Upon allergen presentation by dendritic cells, naïve CD4⁺ T cells differentiate into four distinct populations depending on the cytokine environment: T helper cell type 1 (Th1), Th2, Th17, and regulatory T cells. Asthma is accompanied by the

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induction of antigen-specific Th2 cells and related cytokines such as interleukin (IL)-4, IL-5, and IL-13.^{2–4} Recent studies have shown that Th17 cells and related cytokines, including IL-17A, IL-17F, and IL-22, are also associated with allergic inflammation.⁵ In asthmatic patients, IL-17A concentration is increased in peripheral blood, sputum, and bronchoalveolar lavage fluid (BALF).^{6–8} In experimental models of asthma, Th17 cells enhance not only neutrophilic airway inflammation⁸ but also Th2 cell-mediated eosinophilic airway inflammation.⁹

IL-23, secreted from antigen-presenting cells such as activated macrophages and dendritic cells, is an essential cytokine in the maintenance of Th17 cells, secretion of IL-17A, and memory T-cell proliferation.^{5,9–14} Recently, Wakashin *et al.*⁹ found that IL-23 enhances antigen-induced Th2 cytokine production and eosinophil recruitment in airways. Peng *et al.*¹⁵ reported the activation of IL-23 receptors expressed on Th2 cells promotes Th2 polarization and cytokine production. These results suggest important roles for IL-23 in the development of asthma. Conversely, there are some reports that provide evidence for IL-23 and/or IL-17A as negative regulators of allergic inflammation.^{16,17} IL-23 suppresses inflammatory responses to fungi in the airways in a toll-like receptor (TLR) 6-dependent manner, which includes the activation of T cells, Th2 cytokine production, and granulocyte recruitment.¹⁷

The precise roles of the IL-23/Th17 axis in the regulation of allergic airway inflammation are still controversial. Thus, we performed this study using IL-23 deficient (*IL-23p19*^{−/−}) mice in two different models of asthma.

Methods

Animal preparations

Specific pathogen- and viral antibody-free, 6-week-old, female C57BL/6J (WT) mice weighing between 20 and 25 g were purchased from Charles River Laboratories (Yokohama, Japan). IL-23-deficient (*IL-23p19*^{−/−}) and IL-17A-deficient (*IL-17A*^{−/−}) mice with a C57BL/6 genetic background were developed as previously reported.^{18,19} All animals were housed in a facility in bioBubble® barrier units (bioBubble Inc., Fort Collins, CO) under positive pressure. The experimental protocol was reviewed and approved by the Laboratory Animal Care and Use Committee of Keio University School of Medicine.

Allergen sensitization and exposure

In the ovalbumin (OVA)-induced airway inflammation model, sensitization to OVA was achieved by intraperitoneal injection of 15 µg OVA in an alum solution (Imject® Alum, Pierce Chemical, Rockford, IL) on days 0, 7, and 14. Airway challenge was carried out in a dedicated chamber with aerosolized 1% (w/v) OVA diluted in phosphate buffered saline (PBS) for 20 min on days 21, 22, 23, and 24.

In the house dust mite antigen (HDM)-induced airway inflammation model, mice were actively sensitized against *Dermatophagoides pteronyssinus* antigen (Biostir, Kobe, Japan) by intraperitoneal injection of 10 µg HDM on days 0, 7, and 14. On days 21, 22, and 23, the mice were exposed to HDM (100 µg/body) via the nares. Mice treated with PBS were used as controls.

Bronchoalveolar and peritoneal cavity lavage fluid

Mice were sacrificed by giving an intraperitoneal overdose of pentobarbital 24 h after the final OVA or HDM airway exposure. The trachea was cannulated and the lungs were lavaged with 1.4 ml of cold PBS containing 0.6 mM ethylenediamine tetraacetic acid

(EDTA) to collect bronchoalveolar lavage fluid (BALF). In some animals, the peritoneal cavity was lavaged with 5 ml of PBS/EDTA 3, 6, 12, and 24 h after an OVA or HDM peritoneal injection.

The cells in BALF and the abdominal cavity lavage fluid were counted using a hemocytometer, and white blood cell differential counts were determined on Diff-Quik-stained cytospin slides (Symex, Kobe, Japan).

Cytokine analysis

The concentrations of cytokines in BALF and peritoneal lavage fluid were measured with an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Measurement of serum ova- and hdm-specific IgG₁ antibodies in serum

Blood was collected from the inferior vena cava 24 h after the last challenge of antigens in the airways. OVA IgG₁ was measured using a commercially available ELISA kit (Shibayagi, Gunma, Japan), according to the manufacturer's instructions. HDM-specific IgG₁ antibody in serum was measured by direct ELISA using target antigen-coated plates, biotinylated anti-mouse IgG₁ rat antibody (BD Biosciences, San Jose, CA, USA) and avidin-peroxidase (Sigma–Aldrich, St. Louis, MO, USA). Protein concentration was determined by measuring the optical density at 450 nm after color development with 3,3',5,5'-tetramethylbenzidine Substrate Reagent Set (BD Biosciences), which was stopped by the addition of 1 M H₂SO₄.

Quantitative real-time RT-PCR

Tissue samples were homogenized and total RNA was extracted using the RNeasy Mini kit (QIAGEN, Hilden, Germany). Complementary DNA was generated with SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The expression level of mRNA was 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). We used the $\Delta\Delta$ threshold cycle (ddCt) technique to calculate relative mRNA expression of target genes normalized to levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

Statistical analysis

Data are presented as means $\pm$ SEM. Student's *t*-test and one-way analysis of variance were performed for comparisons of two and three or more groups, respectively. Tukey's tests for comparison with control group and Dunnett's multiple comparison tests for analysis among groups without control group were used as post-hoc tests. Data were analyzed with GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). A *p* value less than 0.05 was regarded as significant.

Results

IL-23 promotes ova-induced asthmatic responses

In order to examine the roles of IL-23, we compared OVA-induced airway inflammation in *IL-23p19*^{−/−} mice with that of wild-type mice (Fig. 1). Although both types of animals exhibited eosinophilic inflammation in response to OVA exposure in the airways, *IL-23p19*^{−/−} mice had less eosinophils in the BALF than wild-type mice (Fig. 1a, b). The amounts of IL-5 and IL-13 in BALF were also reduced by half in *IL-23p19*^{−/−} mice (Fig. 1c, d, *P* < 0.01).

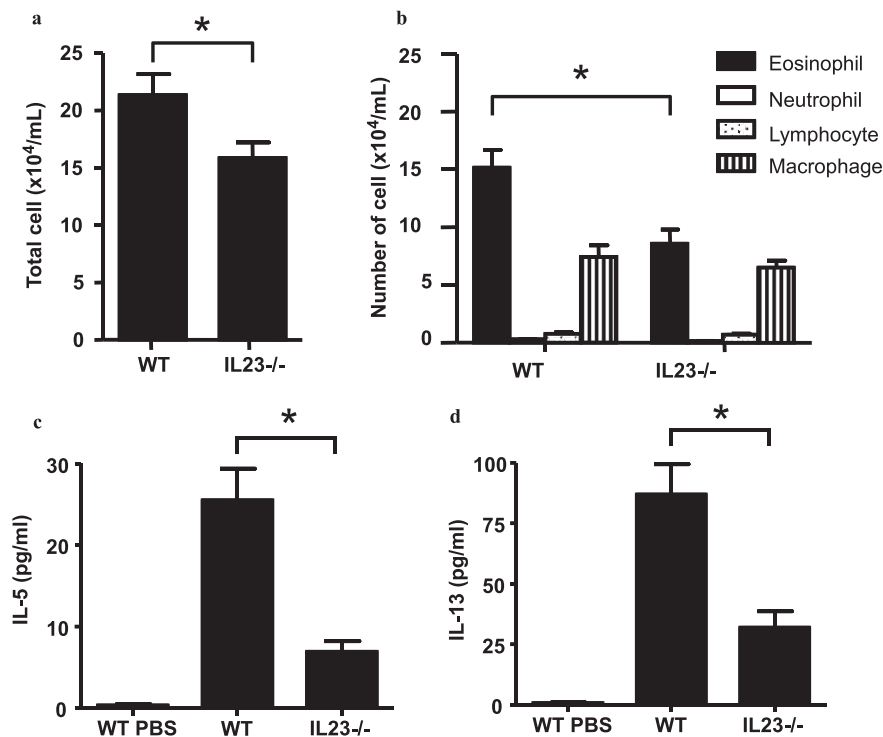


Fig. 1. Ovalbumin (OVA)-induced asthma model. Wild-type (WT) and *IL-23p19^{-/-}* (*IL-23^{-/-}*) mice were sensitized to OVA intraperitoneally and then exposed to aerosolized OVA or phosphate buffered saline (PBS) in the airways. Total (a) and differential (b) cell counts in bronchoalveolar lavage fluid were examined 24 h after the final OVA exposure ($n = 29$ for WT, $n = 29$ for *IL-23^{-/-}*). The concentration of IL-5 (c) and IL-13 (d) in the bronchoalveolar lavage fluid was determined by ELISA ($n = 9-16$). Mean + SEM. * $P < 0.05$ compared to wild-type mice.

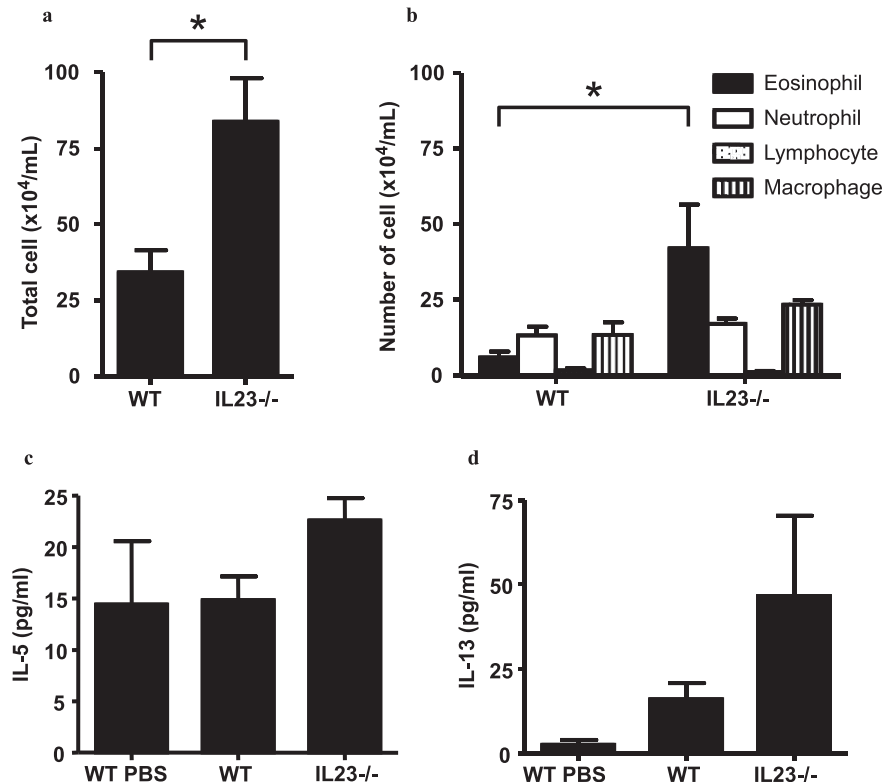


Fig. 2. House dust mite antigen (HDM)-induced asthma model. Wild-type (WT) and *IL-23p19^{-/-}* (*IL-23^{-/-}*) mice were sensitized to HDM intraperitoneally and then exposed to HDM or phosphate buffered saline (PBS) via the nares. Total (a) and differential (b) cell counts in bronchoalveolar lavage fluid were examined 24 h after the final HDM exposure ($n = 7$ for WT, $n = 3$ for *IL-23^{-/-}*). The concentration of IL-5 (c) and IL-13 (d) in the bronchoalveolar lavage fluid was determined by ELISA ($n = 3-7$). Mean + SEM. * $P < 0.05$ compared to wild-type mice.

IL-23 inhibits hdm-induced asthmatic responses

To confirm the asthma-promoting effects of IL-23, we sensitized and exposed mice to HDM antigen, one of the most clinically important allergens, using a similar protocol. In contrast to our expectation, *IL-23p19^{-/-}* mice exhibited significantly higher numbers of eosinophils in the BALF than wild-type animals (Fig. 2a, b, $P < 0.05$). Neutrophilic inflammation was also observed in HDM-treated mice; however, no difference was observed in the number of BALF neutrophils between wild-type and *IL-23p19^{-/-}* mice. Cytokine production induced by HDM was also examined (Fig. 2c,

d). A propensity for both IL-5 and IL-13 to be higher in *IL-23p19^{-/-}* mice was observed although it was not statistically significant.

Peritoneal IL-23 production correlates with antigen-specific serum immunoglobulin levels

We hypothesized that the opposite effects of IL-23 on allergic airway inflammation in the OVA and HDM models were due to site-specific differences in IL-23 expression. We first examined IL-23 synthesis in the peritoneal cavity upon allergen sensitization. Peritoneal injection of OVA with alum adjuvant induced local

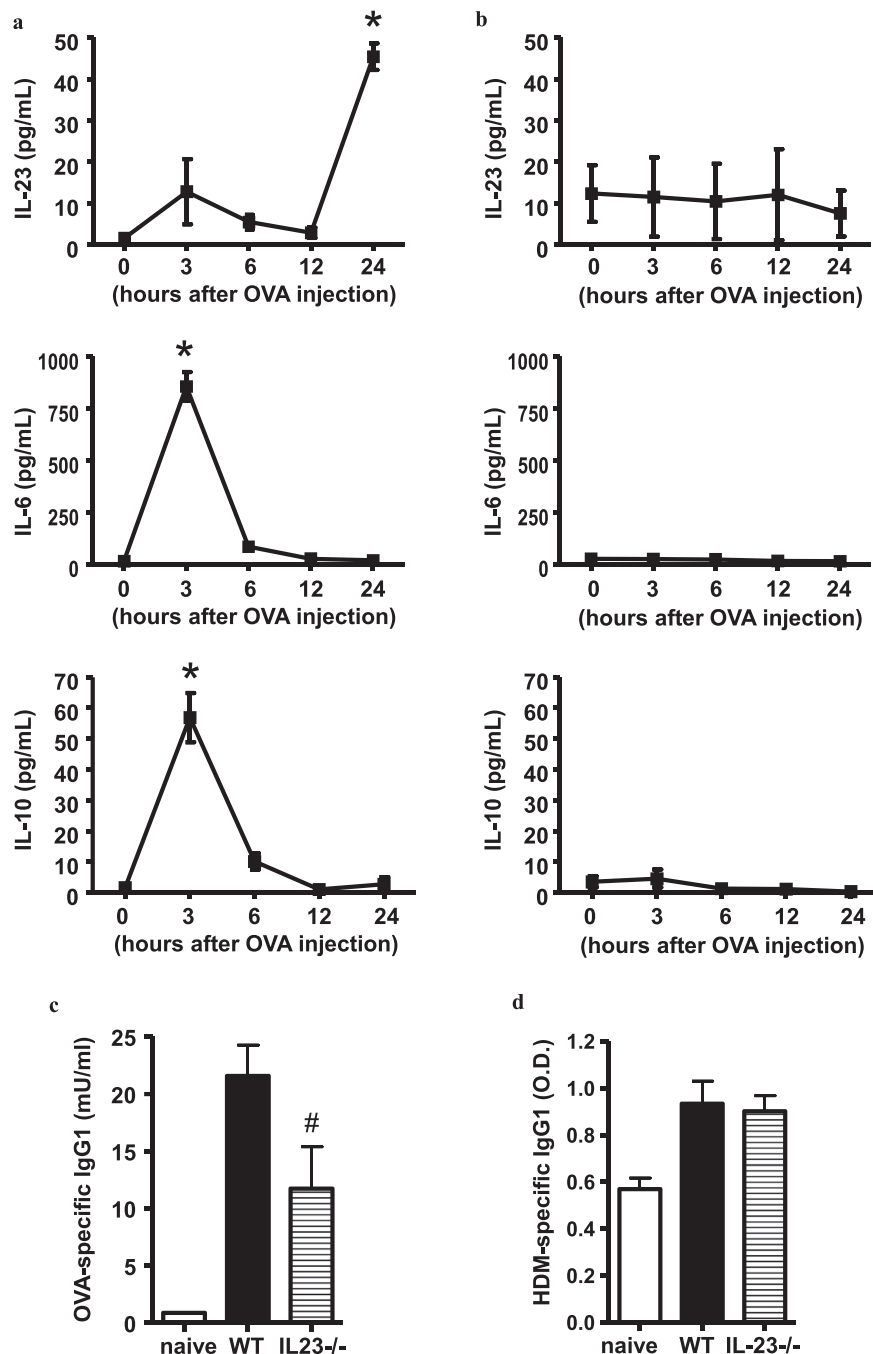


Fig. 3. Production of IL-23 and other cytokines in the peritoneal cavity in response to ovalbumin (OVA, a) or house dust mite antigen (HDM, b) injection. The peritoneal cavity was washed 3, 6, 12, and 24 h after antigen injection ($n = 3$). Mean $\pm$ SEM. * $P < 0.05$ compared to naïve mice. Antigen-specific IgG₁ levels in OVA- (c) or HDM- (d) sensitized mice in sera collected 24 h after antigen challenge were examined in wild-type (WT) and *IL-23p19^{-/-}* (*IL-23^{-/-}*) mice ($n = 6-12$). Mean $\pm$ SEM. # $P < 0.05$ compared to wild-type mice.

synthesis of IL-23 in a biphasic manner, peaking at 3 and 24 h, while HDM did not increase IL-23 levels in the peritoneal cavity (Fig. 3a, b). IL-6 and IL-10 were also released in the peritoneal cavity 3 h after OVA administration.

Repeated injection of antigen into the peritoneal cavity for sensitization, either OVA in conjunction with alum or HDM, induced the production of allergen-specific IgG₁ in serum. However, the serum levels of OVA-specific IgG₁ were reduced in *IL-23p19*^{-/-} mice, whereas HDM-specific IgG₁ showed no difference between *IL-23p19*^{-/-} and wild-type mice (Fig. 3c, d). These results also support the idea that sensitization to OVA, but not to HDM, is promoted by IL-23.

Pulmonary expression of IL-23 and IL-17A negatively regulates asthmatic responses

Next, we investigated gene expression of *IL-23p19* in the airways in response to OVA or HDM challenge. Exposure to aerosolized OVA did not alter *IL-23p19* mRNA expression in the lungs (Fig. 4a), whereas nasal administration of HDM induced and maintained

enhanced expression of pulmonary *IL-23p19* mRNA for up to 10 days (Fig. 4b). Induction of IL-23 in the airways was associated with concomitant expression of IL-17A in the lungs, which negatively regulated eosinophilic inflammation. *IL-17A* mRNA was induced by administration of HDM in wild-type, but not in *IL-23p19*^{-/-} mice (Fig. 4c, d). IL-17A deficiency induced a more prominent recruitment of eosinophils in the airways after HDM challenges as IL-23 deficiency did (Fig. 4e).

Discussion

In the present study, we demonstrate opposing roles of IL-23 in two murine models of asthma; IL-23 deficiency protected mice from OVA-induced allergic responses, but, rather, exacerbated HDM-induced eosinophilic inflammation.

The pro-allergic effects of IL-23 on the promotion of Th2 cell differentiation and related cytokine production are well described in murine models of asthma using OVA as the allergen.^{9,15,20–22} Administration of anti-IL-23-neutralizing antibodies,^{9,22} silencing of IL-23 expression by a small hairpin RNA²¹, a peptide-based

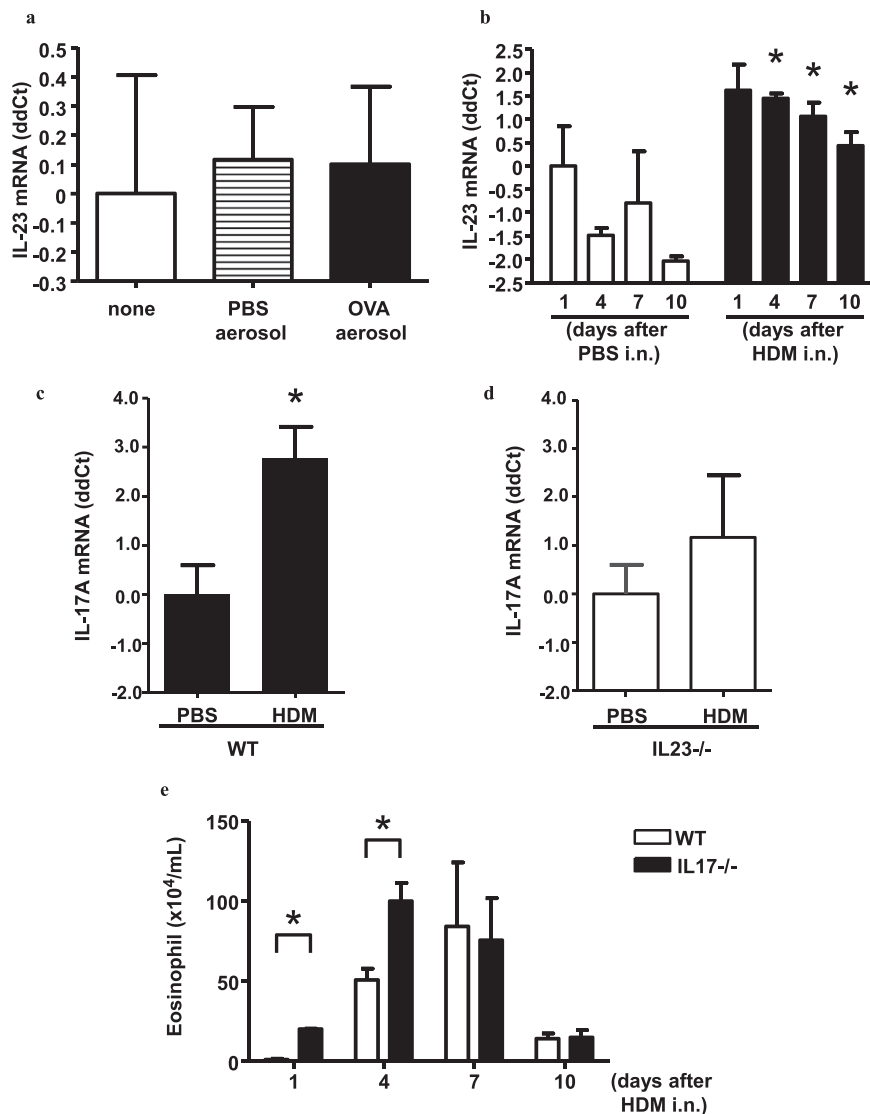


Fig. 4. *IL-23p19* mRNA expression in the lungs. Mice were sensitized to OVA (a) or HDM (b) then exposed to phosphate buffered saline (PBS) or antigen as indicated (n = 3 for each group). *IL-17A* mRNA expression in the lungs of wild-type (c) or *IL-23p19*^{-/-} (d) mice after a single exposure to HDM (n = 3). *P < 0.05 compared to the PBS group. (e) Eosinophil count in bronchoalveolar lavage fluid from wild-type (open bars) or *IL-17A*^{-/-} (closed bars) mice. Mean + SEM. *P < 0.05 compared to wild-type mice.

vaccine,²⁰ and the introduction of genetic mutation¹⁵ in IL-23 all suppress the induction of OVA-specific IgE, eosinophilic airway inflammation, and cytokine synthesis including IL-4, IL-5, IL-13, and IL-17. The role of IL-23 is further supported by a clinical observation that serum IL-23 levels are inversely correlated with lung functions in asthmatic children.²³ These findings are in accordance with our data in the OVA-induced asthma model; eosinophil accumulation and IL-5/IL-13 synthesis in the lungs as well as OVA-specific IgG₁ production were significantly suppressed in IL-23p19^{-/-} mice.

In contrast, HDM-driven allergic airway inflammation was rather enhanced in the absence of IL-23 or IL-23-dependent synthesis of IL-17A (Figs. 2, 4). Other reports have shown that IL-23 and IL-17A negatively regulate eosinophilic airway inflammation under certain conditions, especially in the presence of infectious pathogens such as rhinovirus or fungi, which induce robust IL-23 expression in the lungs.^{17,24} In these models, blocking IL-17A signals enhances eosinophilic airway inflammation, as observed in our HDM-induced model.^{17,24,25} Furthermore, exogenously-administered IL-17A directly acts as a negative regulator of allergic asthma.^{16,26,27} Importantly, the dose and cellular source of IL-17A is crucial to determine whether the cytokine acts as a positive or a negative regulator in allergic inflammation. Higher doses of IL-17A or $\gamma\delta$ T cells suppress eosinophil recruitment to the airways, whereas lower doses of IL-17A or $\alpha\beta$ T cell-derived cytokines co-operate with Th2 cytokines to enhance airway eosinophilia.^{26,28} These data are consistent with the results observed in the HDM-induced model that was associated with enhanced synthesis of IL-17A in the lungs.

Targeting IL-23 and IL-17A has been suggested as a therapeutic approach for severe cases of asthma refractory to corticosteroids. Clinical asthma, however, is not induced by sensitization to a simple protein antigen, such as OVA, but by exposure to protease antigens, such as HDM and fungi, or in association with a viral infection that might induce high levels of IL-23/IL-17A expression and reduce Th17 cells in the airways. That may explain why systemic administration of an anti-IL-17A antibody failed to improve asthma symptoms in a clinical trial.²⁹ A novel approach that selectively inhibits IL-23 signaling toward Th17 differentiation or proliferation could be an effective therapeutic approach to overcome this problem.

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Conflict of interest

YS received research funding from Merck, Sharp, and Dohme (MSD). KA received research funding from Astellas Pharma; honoraria as lecture fees from Astellas Pharma, GlaxoSmithKline (GSK) and MSD. The rest of the authors have no conflict of interest.

Authors' contributions

KA, YS, and RO designed the study and wrote the manuscript. AY, SF, TT, and TB also contributed to the study design. SK, KM, and KF contributed to data collection and interpretation of the results. All authors read and approved the final manuscript.

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