



Critical role of interleukin-23 in development of asthma promoted by cigarette smoke

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Abstract

It has been recently reported that cigarette smoke exposure during allergen sensitization facilitates the development of allergic asthma; however, the underlying mechanisms remain elusive. We evaluated the role of interleukin (IL-23) in a cigarette smoke extract (CSE)-induced *Dermatophagoides pteronyssinus* (Dp)-allergic asthma mouse model. BALB/c mice were exposed to CSE during allergen sensitization period. Anti-IL-23p19 or IL-23R antibody was administered during the sensitization period. And we evaluated several immunological responses. The expression of IL-23 and IL-23 receptor (IL-23R) was examined in lung tissue. IL-23 and IL-23R expression was increased in the airway epithelium of Dp/CSE co-administered mice. CSE administration during the sensitization promoted Dp-allergic sensitization and the development of asthma phenotypes. Additionally, the proportion of innate lymphoid type 2 cells (ILC2) was also increased by CSE and Dp co-instillation. Anti-IL-23 or IL-23R antibody treatment during allergen sensitization significantly diminished phenotypes of allergic asthma and the ILC2 population. The levels of IL-33 and thymic stromal lymphopoietin (TSLP) were also significantly reduced by anti-IL-23 or IL-23R antibody treatment. IL-23 may thus play a significant role in cigarette smoke-induced allergic sensitization and asthma development. Clinically, the increase in allergen sensitization due to cigarette exposure causes onset of asthma, and IL-23 may be important in this mechanism.

Key messages

- IL-23 and IL-23R expression was increased in the lung epithelium of Dp and CSE co-exposed mice during sensitization period.
- The population of ILC2s was increased in Dp and CSE co-exposed mice during sensitization period.
- Anti-IL23 or IL-23R antibody treatment with co-administration of CSE and HDM during sensitization period significantly suppresses ILC2.
- In vitro, IL-23 blockade in Dp and CSE-stimulated epithelial cells suppressed IL-13 expression in ILC2.

Keywords IL-23 · IL-23 receptor · Cigarette smoke · House dust mite

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Introduction

Antigen sensitization is known to be very important in the development of asthma. And environmental factors are known to enhance antigen sensitization [1]. Epidemiologic studies have shown that cigarette smoke exposure to be one among various environmental factors that can increase development of asthma [2–4]. Several studies have reported that exposure to cigarette smoke may increase antigen susceptibility and lower the threshold for antigen sensitization [5–7]. However, there are insufficient studies on the mechanism underlying asthma development by smoking.

A recent study demonstrated enhancement of asthma development by increased sensitization to antigens upon only a short-term exposure to smoking. In this study, the authors established a model of an increase in development of asthma by smoke exposure during sensitization [8]. However, this study insufficiently explained the major mechanisms involved in asthma or the factors that could inhibit this phenotype.

Innate lymphoid type 2 cells (ILC2) have been recently shown to be the main cells involved in the innate immune response, which plays a very important role in asthma [9, 10]. Since allergic asthma aggravated by smoking is known to involve antigen sensitization as an important step [11], the innate immune response is thought to be important in this process. Therefore, ILC2 might also be involved in smoke-induced sensitization.

IL-23 is a major pro-inflammatory cytokine and plays an important role in autoimmune diseases [12–14]. Additionally, IL-23 is well-known as a major causative agent of psoriasis in relation to allergic inflammation [15–17]. IL-23 is also reportedly regulating Th2 cells as an adaptive immune response in asthma [18–20]. However, the role of IL-23 in asthma development is unclear. In our recent study, we suggested that IL-23 may play an important role in antigen sensitization in the mouse model of house dust mite (HDM)-induced asthma [21].

In the present study, we attempted to clarify the detailed mechanisms underlying enhancement of asthma development by smoking exposure. We hypothesized that IL-23 play a role in the development of allergic asthma by cigarette smoke in mice. To confirm this hypothesis, we evaluated the expression and role of IL-23 at the developmental stage of cigarette smoke-exposed antigen sensitization.

Materials and methods

Animals

Six-week-old female BALB/C mice (18–20 g) were purchased from Orient Bio (Seoul, Korea). All experiments were

performed with the approval of the Institutional Animal Care and Use Committee of the Institute (IACUC) of Seoul National University. The approval number is SNU-160615-5-4.

Preparation of cigarette smoke extract (CSE)

The method for making these measurements is provided in an online data supplement.

Exposure of CSE with low-dose HDM (Dp)

The mice were sensitized intranasally 10 µg HDM extract (*Dermatophagoides pteronyssinus*; Dp 1 1.88 µg/mg protein, PROLAGEN, Korea) with or without 40% CSE.

ELISA assay, immunohistochemistry stain, and immunofluorescence stain in lung tissue

The method for making these measurements is provided in an online data supplement.

Development of cigarette smoke exposure-induced house dust mite model and anti-IL-23 or IL-23R antibody treatment

Goat anti-mouse IL-23p19 Ab (2 µg), rat anti-mouse IL-23R Ab (2 µg) were used to block IL-23 or IL-23R bioactivity in mice. Both antibodies were purchased by R&D systems. Goat or rat IgG Ab was also treated in mice as control. This dose of anti-IL-23p19 Ab has been shown to successfully neutralized IL-23 or IL-23R activity in vivo [22, 23]. CSE or Dp was resuspended in phosphate-buffered saline (PBS) and mice were instilled by intranasal injection. Two antibodies were treated by intravenous route before the instillation of Dp or/and CSE (Fig. 2a).

Measurement of methacholine hyperresponsiveness, analysis of bronchoalveolar lavage fluid, histopathology and analysis of Th2 cells, and type 2 innate lymphoid cells (ILC2) in lung tissues

The method for making these measurements is provided in an online data supplement.

Evaluation of DC activation in lung draining lymph node (LDLN)

Mice were sacrificed 24 h after last sensitization with Dp with or without CSE. The LDLNs were removed

and processed. The percentage of DCs was determined by flow cytometry as the fraction of MHCII⁺ and CD86⁺ in CD11c⁺ cells.

Sorting of type 2 innate lymphoid cell (ILC2) and co-culture of MLE12 cells and ILC2 in the presence of transwell inserts

The method for making these measurements is provided in an online data supplement.

Analysis of data using flow cytometry

Samples were analyzed on LSRII (BD Biosciences, San Jose, CA, USA) using FlowJo ver. 10 software (TreeStar).

Statistics

Results are expressed as mean $\pm$ standard deviation (SD), and statistical differences among groups were assessed using Kruskal-Wallis test or Mann-Whitney test. For multiple comparisons, Kruskal-Wallis test was used initially. As significant differences were found, Mann-Whitney test was used for the comparison of statistical difference between two groups. Statistical

analyses were performed using GraphPad Prism 4.01 (GraphPad Software, La Jolla, CA, USA). *P* values < 0.05 were considered significant.

Results

Increased IL-23 expression in the lung epithelium of Dp and cigarette smoke extract-exposed mice during the sensitization period

Cigarette smoke exposure during sensitization has been shown to be important for subsequent asthma development [8]. Therefore, in this experiment, we considered that IL-23 might play an important role in sensitization by Dp, which is increased by smoking. We first administered low-dose Dp and CSE during the sensitization period and measured the expression of IL-23 (Fig. 1a). ELISA of crushed lung supernatants showed increased expression of IL-23 in whole lung tissues from Dp/CSE-exposed mice compared to mice exposed to Dp alone (Fig. 1b). Moreover, immunohistochemistry and immunofluorescence staining (green) and of lung sections showed increased IL-23 expression in airway epithelium of Dp/CSE-exposed mice compared to that in mice that received Dp or CSE alone (Fig. 1c–e).

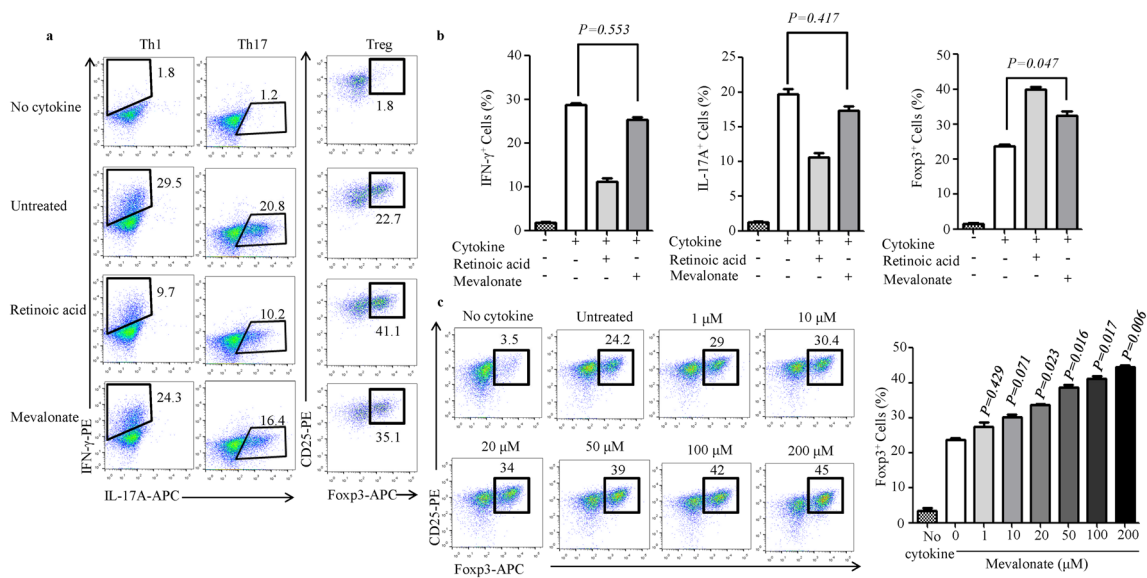


Fig. 1 The expression of IL-23 in lung of mice simultaneously exposed to PBS or Dp and 3 days of cigarette smoke extract (CSE). **a** Experimental protocol. The expression of IL-23 from lung was detected after instillation of Dp with or without CSE. **b** The protein level of IL-23

in crushed lung was detected using ELISA. **c** The expression of IL-23 in lung was detected using immunohistochemistry staining (IHC). **d** IL-23 IHC quantification. **e** The expression of IL-23 in lung was detected using immunofluorescence staining (IF, green). **p* < 0.05, ***p* < 0.01

Airway inflammation and AHR was decreased in anti-IL-23 Ab-treated mice during Dp/CSE sensitization

First, we sought to determine IL-23 secretion and the effects of anti-IL-23 Ab treatment after sensitization. Protein levels of IL-23 in lung tissues were significantly increased upon exposure to CSE with Dp in mice, and were significantly reduced in anti-IL-23 Ab-treated mice (Fig. S1A). Immunofluorescence staining showed that IL-23 expression in the airway epithelium was decreased in anti-IL-23 Ab-treated mice (Fig. S1B) compared to that in mice exposed to CSE intranasally with Dp (Fig. 1e).

To examine the role of IL-23 during sensitization in Dp/CSE-induced asthma, we administered anti-IL-23p19 Ab to the mice (Fig. 2a). In methacholine challenge tests, AHR was significantly increased upon intranasal exposure of CSE with Dp in mice. However, AHR was significantly reduced by anti-IL-23p19 Ab treatment (Fig. 2b). The number of inflammatory cells including eosinophils in the BALF, the recruitment of inflammatory cells into the lungs and Dp-specific IgG1 in the serum were increased upon intranasal exposure to CSE with Dp in mice, but were significantly reduced by anti-IL-23p19 Ab treatment (Fig. 2c–e).

Th2 cells and IL-13-producing ILC2 were decreased in anti-IL-23-treated mice during sensitization

Next, we investigated changes of Th2 cells in this mouse model, and observed a significant increase in IL-13 and IL-5 expression in CD4⁺ T cells. Thus, populations of IL-13-positive and IL-5-positive CD4⁺ T cells were increased upon exposure to CSE with Dp in mice. However, this increase in Th2 cells was

significantly reduced in anti-IL-23 Ab-treated mice (Fig. 3a–b). Recent studies demonstrated that HDM could affect ILC2-dependent production of Th2 cytokines [24, 25]. Additionally, ILC2 involvement has been suggested in the development of allergic asthma [26]. To examine the kinetics of ILC2 responses, we assessed ILC2 frequencies after sensitization (day 4), on day 14, and after challenge (day 25). We found that there were few ILC2 and no changes at day 4, whereas the number of ILC2 increased upon exposure to CSE with Dp at days 14 and 25 (Fig. S2).

Therefore, we also measured the population of ILC2 after challenge, which the time point was used for all phenotypic analyzes. We examined the effects of IL-23 blockade on lung ILC2 and found that anti-IL-23 Ab treatment also significantly reduced the population of IL-13-producing ILC2 in Lin[−]ST2⁺CD25⁺ cells (Fig. 3c).

Effects of IL-23 on dendritic cell (DC) activation in lung draining lymph nodes and ILC2-related innate cytokines in the lungs

We also examined the changes in DC activation in the lung draining lymph nodes to determine the activation changes of DCs that increase the adaptive immune response to the antigen. We found that the increased population of MHCII+CD86+ in CD11c cells was significantly reduced by anti-IL-23p19 Ab treatment (Fig. 3d). To access the mechanism of this reduction of DC activation, we measured the level of GM-CSF. The protein level of GM-CSF in lung tissue was increased upon

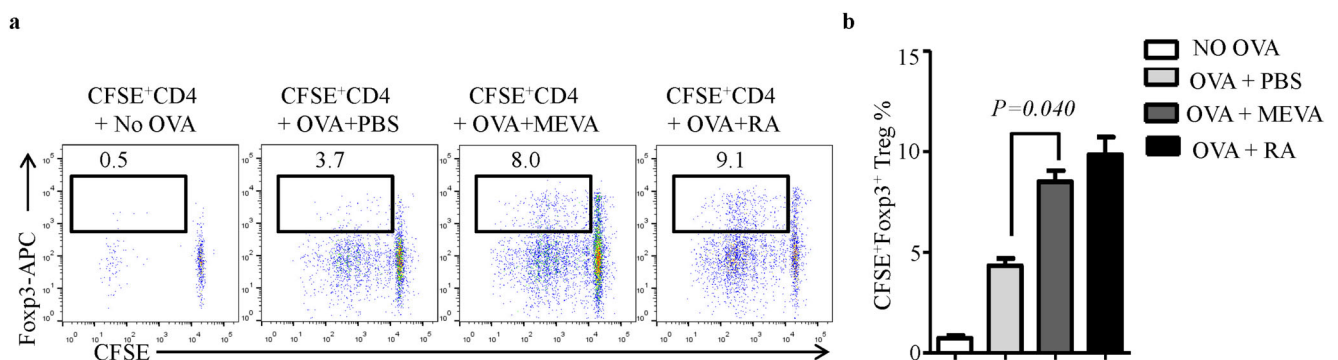


Fig. 2 The evaluation of asthmatic phenotypes in anti-IL-23 Ab-treated mice during sensitization period. **a** Experimental protocol for house dust mite and cigarette smoke extract induced asthma model of study. **b** Methacholine hyperresponsiveness was measured 24 h after the last challenge. **c** The numbers of eosinophils and neutrophils in BALF. **d** Lung

histology after the last challenge (a: PBS, b: CSE, c: Dp, d: Dp/CSE, e: CSE + anti-IL-23 Ab, f: Dp + anti-IL-23 Ab, g: Dp/CSE + anti-IL-23 Ab, H&E stain, $\times 200$). **e** Serum Dp-specific IgG1 was evaluated after the last challenge. OD, optical density; * $p < 0.05$; ** $p < 0.01$

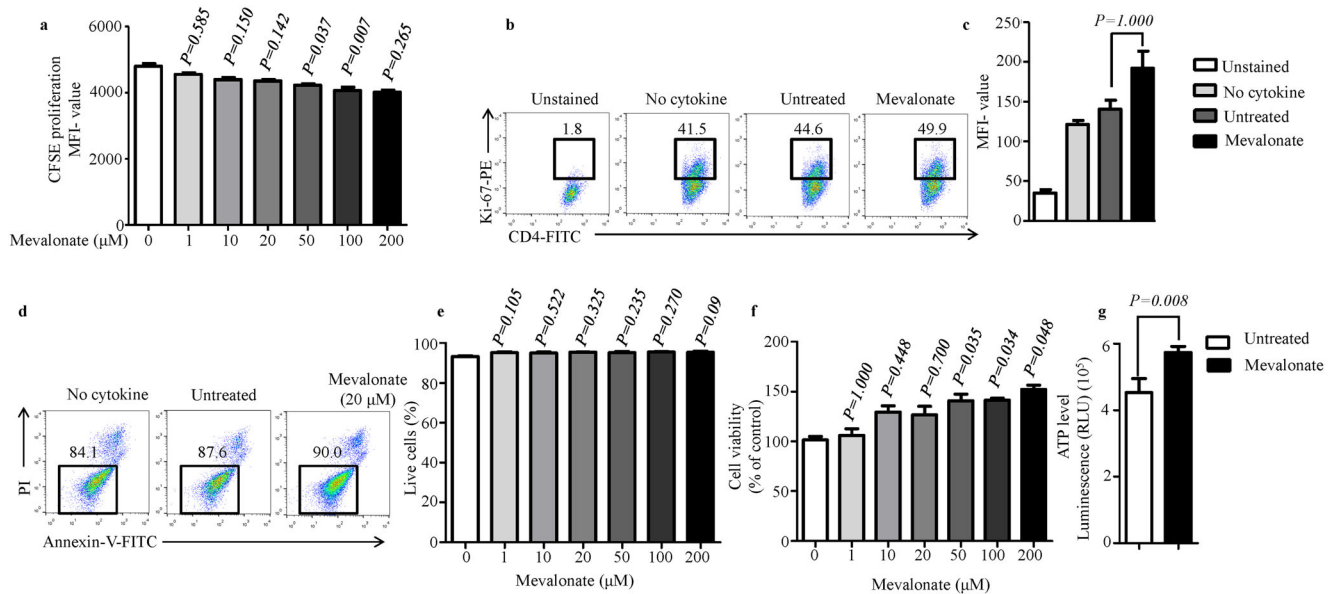


Fig. 3 The population of Th2 cells, IL13 + ILC2 after challenge and the numbers of DCs, the level of innate pro-Th2 cytokines after the last sensitization. **a–c** IL13 or IL5-produced CD4⁺ T cells and IL13-produced type 2 innate lymphoid cells from lung were evaluated after the last challenge in anti-IL-23 Ab-treated mice during sensitization. After treatment of anti-IL-23 Ab in the sensitization period, mice were

sacrificed 24 h after the last sensitization. After sacrifice, lung draining lymph nodes were isolated from mice. **d** The numbers of MHCII+ CD86+ cells in CD11c+ DCs were determined using flow cytometry. **e–f** The levels of IL-33 and TSLP in the supernatants of crushed lungs were determined using ELISA; * $p < 0.05$; ** $p < 0.01$

exposure to CSE with Dp in mice, which was significantly reduced in anti-IL-23 Ab-treated mice (Fig. S3). To explain the mechanisms underlying ILC2 reduction by IL-23 blockade during the sensitization period, we measured changes in IL-33 and thymic stromal lymphopoietin (TSLP). Protein levels of IL-33 and TSLP in lung tissues were significantly increased upon exposure to CSE with Dp, and were significantly reduced in anti-IL-23 Ab-treated mice (Fig. 3e–f).

Increased IL-23R expression in the lung epithelium of Dp and CSE-exposed mice during the sensitization period

IL-23 receptor (IL-23R) is important for the functioning of the IL-23 signaling pathway [27]. Additionally, IL-23 may regulate innate cytokines by an autocrine mechanism in bronchial epithelial cells [21]. Therefore, we hypothesized that IL-23R might also play a role in the development of allergic asthma promoted by CSE exposure. Interestingly, immunohistochemistry of lung sections showed that IL-23R expression was increased in Dp/CSE-exposed mice compared to that in Dp- or CSE-exposed mice (Fig. 4a–b). Therefore, to examine the role of IL-23 signaling, we assessed phenotypic changes after challenge with neutralizing IL-23R during the

sensitization period. Anti-IL-23R Ab treatment resulted in a reduction of increased AHR and fewer inflammatory cells, including eosinophils, from BALF (Fig. 4c–d).

IL-13-producing CD4⁺ T cells and IL-13-producing ILC2 were decreased in anti-IL-23R-treated mice during sensitization

We assessed changes in serum Dp-specific IgG1 levels, IL-13-producing ILC2, and IL-13-producing CD4⁺ cells in mice that received IL-23R inhibition (Fig. 5a–c).

Additionally, we examined whether activation of DCs in lung draining lymph nodes and levels of innate pro-Th2 cytokines in the lung were altered by anti-IL-23R Ab treatment. We found that DC activation and levels of IL-33 and TSLP in whole lung tissues were significantly reduced by anti-IL-23R Ab treatment (Fig. 5d–f).

IL-33 and TSLP expression in the lung epithelium was decreased in anti-IL-23 or anti-IL-23R-treated mice during sensitization

In the previous experiment, we observed changes in IL-33 and TSLP levels in crushed lung. Next, we measured the

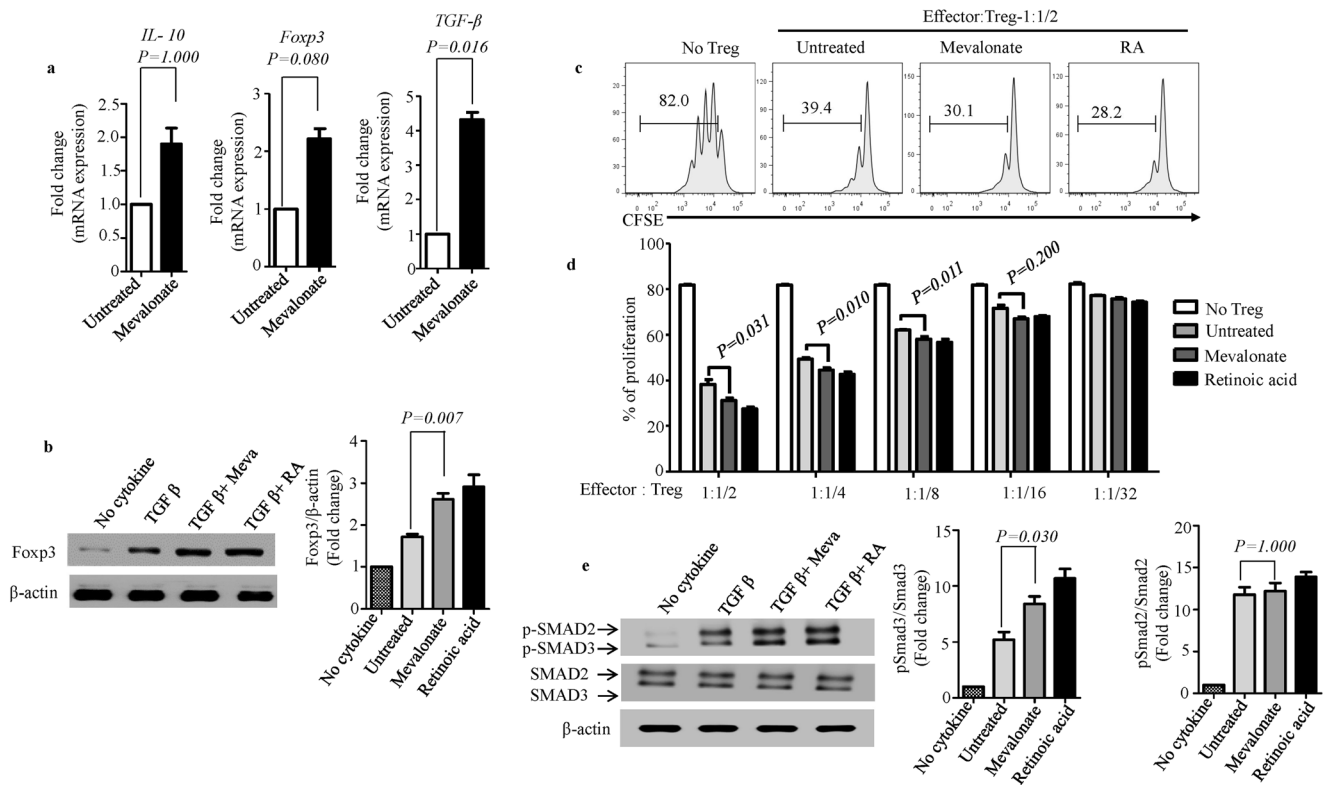


Fig. 4 The expression of IL-23R in lung of mice after sensitization and asthmatic phenotypes after challenge in anti-IL-23R Ab-treated mice during sensitization period. The expression of IL-23R in lung of mice simultaneously exposed to PBS or house dust mite and 3 days of cigarette smoke extract. **a** The expression of IL-23R in lung was detected using

immunohistochemistry staining (IHC). **b** IL-23R IHC quantification. After treatment of anti-IL-23R Ab in the sensitization period, mice were sacrificed 24 h after the challenge. **c** Methacholine hyperresponsiveness was measured 24 h after the last challenge. **d** The number of eosinophils and neutrophils in BALF. * $p < 0.05$, ** $p < 0.01$

expression of IL-33 and TSLP in airway epithelial cells from mouse lung sections. Expression levels of IL-33 in the basal cellular layer of epithelium from lung sections were increased after exposure to CSE with Dp compared to those in mice treated with CSE or Dp alone. However, the increased expression of IL-33 was decreased in anti-IL-23 or IL-23R Ab-treated mice (Fig. 6a–b). TSLP expression was also increased in the epithelial cells from lung sections upon exposure to CSE with Dp in mice. However, the increased expression of TSLP was decreased in anti-IL-23 or IL-23R Ab-treated mice (Fig. 7a–b).

IL-23 blockade in Dp/CSE-stimulated epithelial cells suppressed IL-13 expression in ILC2

Finally, we determined whether IL-23 inhibition affected changes in Dp/CSE-stimulated lung epithelial cells and cytokine levels in ILC2. We first measured the expression levels of IL-23, IL-33, and TSLP in MLE12 cells. IL-23 expression was increased in Dp/CSE-stimulated cells (Fig. 8a). In IL-23 siRNA-pretreated MLE12 cells stimulated with Dp/CSE, the mRNA expression levels of IL-33 and TSLP were decreased compared to those in control Dp/CSE-stimulated MLE12 cells

(Fig. 8b). We also confirmed the corresponding changes in cytoplasmic protein levels (Fig. 8c). To sort ILC2, we used several Dp-treated mice. Pooled lung cells from mice were stained with lineage cocktail, ST2, and ICOS antibodies (Fig. 8d–e). After co-culture of IL-23 siRNA pre-stimulated and Dp/CSE-treated MLE12 cells with ILC2, the levels of IL-13 in ILC2 were measured and found to be significantly reduced compared to the levels in Dp/CSE-stimulated MLE12 cells (Fig. 8f).

Discussion

In this study, we found that exposure to cigarette smoke extract during Dp sensitization promoted the development of Dp-allergic asthma, especially with a low antigen concentration. Several epidemiological studies have shown that cigarette smoke can increase sensitization to HDM allergens [11]. However, mechanistic studies using mouse models, including knowledge of the suppression mechanism, have remained insufficient. Lanckacker and colleagues have developed a mouse model showing that short exposure to cigarette smoke can increase sensitization to HDM allergen. In our mouse model, the

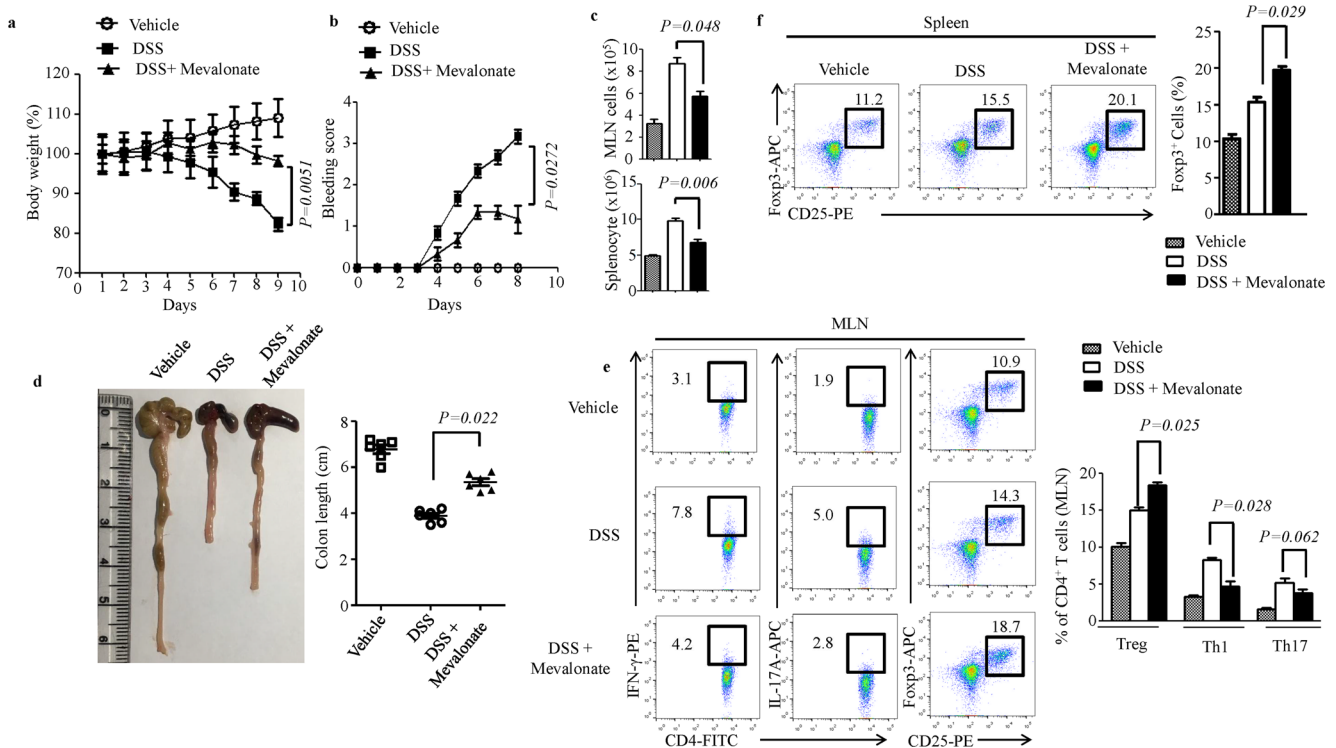


Fig. 5 The population of IL13+ CD4+ T cells, IL13 + ILC2 after challenge and the numbers of DCs, the level of innate pro-Th2 cytokines after the last sensitization. **a** Serum Dp-specific IgG1 was evaluated after the last challenge in anti-IL-23R Ab-treated mice during sensitization. **b–c** IL13-produced CD4+ T cells and IL13-produced type 2 innate lymphoid cell from lung were evaluated after the last challenge in anti-IL-23R Ab-

treated mice during sensitization. After treatment of anti-IL-23R Ab in the sensitization period, mice were sacrificed 24 h after the last sensitization. After sacrifice, lung draining lymph nodes were isolated from mice. **d** The numbers of MHCII+ CD86+ cells in CD11c + DCs were determined using flow cytometry. **e–f** The levels of IL-33 and TSLP in the supernatants of crushed lungs were determined using ELISA. * $p < 0.05$, ** $p < 0.01$

asthma phenotype increased as a consequence of exposure to cigarette smoke extract during a low-dose Dp sensitization period. It can also be considered as a model to explain the hypothesis that cigarette smoke can increase susceptibility for antigen sensitization. However, since the establishment of this model [8], few studies have focused on the detailed mechanism or factors that can inhibit asthma development.

ILC2 have recently been shown to be a critical cell type in the innate immune response in asthma [28]. It is known that ILC2 can secrete IL-13, IL-5, and IL-4. Among these cytokines, IL-13-positive ILC2 is known to be closely related to asthma pathogenesis [29]. We hypothesized that ILC2 could be involved in asthma development in mice following exposure to cigarette smoke, along with the previously known increase in Th2 cytokines and DC activation. Indeed, the population of IL-13-positive ILC2 was increased in Dp/CSE-treated mice. Therefore, ILC2 along with Th2 cells maybe important in the antigen-induced mouse model of asthma promoted by cigarette smoke.

IL-23 is a major pro-inflammatory cytokine and is known to be a very important pathology inducer, especially in autoimmune diseases and psoriasis [30, 31]. It has been studied for its role in asthma and well-known its effects on adaptive immune responses [18, 19]. Recently, we reported the

mechanistic roles of IL-23 in the development of allergic asthma. We showed that IL-23 signaling in antigen-induced asthma may also influence activation of ILC2 in mice, thereby affecting innate immune responses as well as adaptive immune responses. Notably, IL-23 secreted by bronchial epithelial cells was suggested to play a significant role in asthma development [21]. In this present study, we observed a significant increase in Th2 cells and ILC2 in the cigarette smoke-enhanced antigen-induced mouse model, thereby suggesting possible roles of IL-23 in this pathology induced by cigarette smoke exposure. To confirm this hypothesis, we measured IL-23 levels after sensitization with low-dose Dp and CSE, and found that IL-23 protein levels in crushed lungs and expression in airway epithelial cells of lung sections were increased compared to those in groups treated with Dp or CSE individually.

We also investigated whether IL-23R expression is functionally important for IL-23 expression in airway epithelial cells. We also found that IL-23R expression was increased in mouse lung epithelial cells from Dp/CSE-exposed mice. This represents the first report of IL-23R expression in mouse airway epithelial cells from lung sections.

Several studies have shown that IL-23R expression is critical in autoimmune diseases and psoriasis [31, 32]. However, only a few studies have investigated its expression and roles in

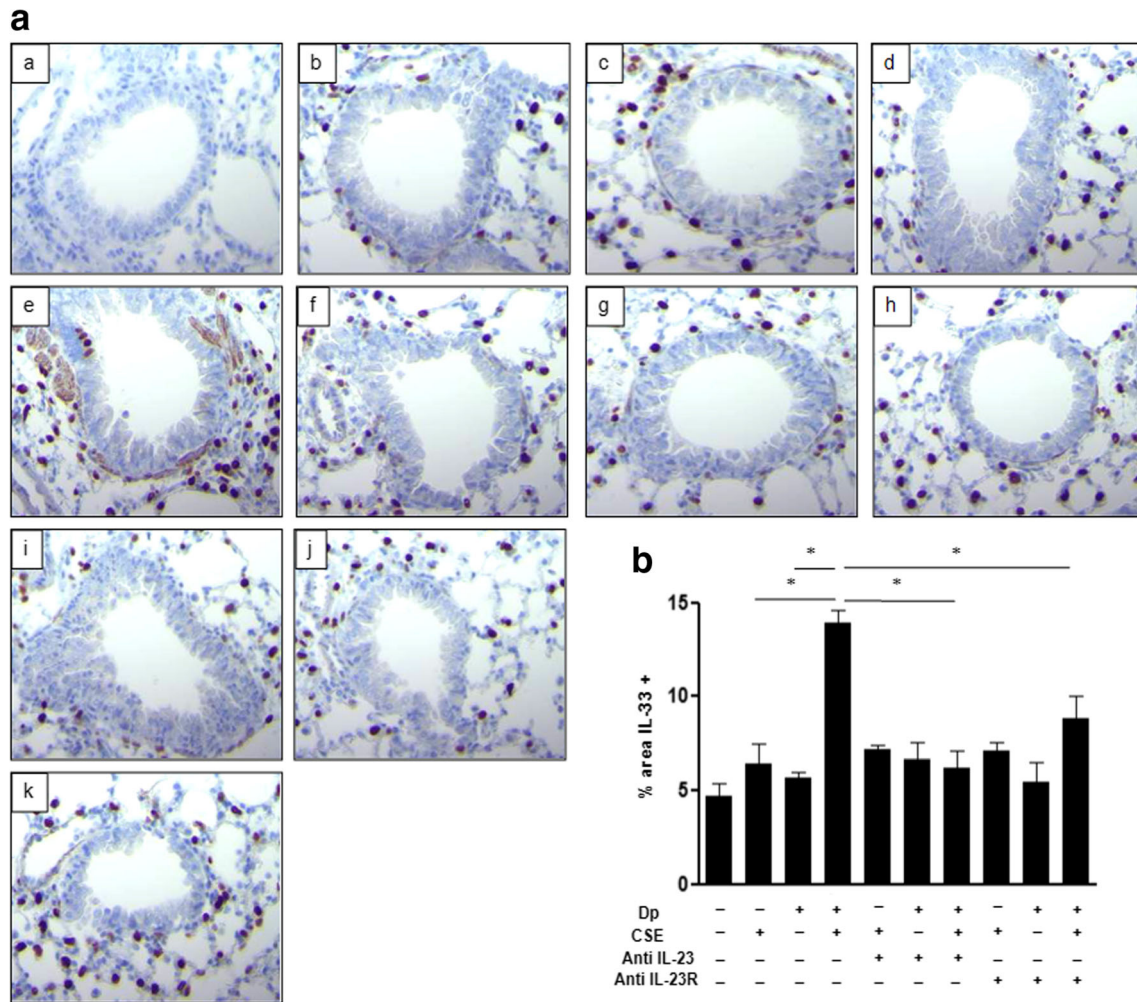


Fig. 6 The expression of IL-33 from lung section in anti-IL-23 Ab or anti-IL-23R Ab-treated mice during sensitization period. After treatment of anti-IL-23 or IL-23R Ab in the sensitization period, mice were sacrificed 24 h after the last sensitization. **a** The expression of IL-33 in lung was detected using immunohistochemistry staining (IHC) (a: isotype

control, b: PBS, c: CSE, d: Dp, e: Dp/CSE, f: CSE + anti IL-23 Ab, g: Dp + anti IL-23 Ab, h: Dp/CSE + anti-IL-23 Ab, i: CSE + anti-IL-23R Ab, j: Dp + anti-IL-23R Ab, k: Dp/CSE + anti-IL-23R Ab, $\times 400$). **b** IL-33 IHC quantification. $*p < 0.05$

the context of asthma. Therefore, we hypothesized that both IL-23 and IL-23R might play roles during sensitization to antigen and cigarette smoke exposure. To validate this hypothesis, anti-IL-23 or IL-23R antibodies were administered during the sensitization period to Dp/CSE-exposed mice. We observed that several features of allergic asthma, such as AHR, lung infiltration of eosinophils, Th2 cells, and ILC2, were decreased by anti-IL-23 or IL-23R treatment. These findings suggest non-redundant roles for IL-23 in the allergic sensitization process promoted by cigarette smoke exposure.

It is known that the antigen uptake, DC migration, and DC activation play important roles in the subsequent increase of Th2 cells [33]. Notably, GM-CSF is a well-characterized innate cytokine that plays a role in Th2 inflammation by the recruitment and activation of DCs [34]. Additionally, IL-33, TSLP, and IL-25 play

major roles in ILC2 activation. IL-33 and TSLP are known to induce ILC2 activation when they are secreted by airway epithelial cells [26, 35]. In this study, we considered that the reduction in Th2 cells and ILC2 induced by IL-23 or IL-23R inhibition could be the mechanism underlying DC and innate cytokines changes. By examining these changes after sensitization alone, we confirmed the reduction of DC activation, secretion of GM-CSF, and reduced expression levels of IL-33 and TSLP in airway epithelial cells. This represents the first study to examine the increased expression levels of IL-33 and TSLP in mouse airway epithelial cells induced by sensitization to antigen and cigarette smoke. This is also the first study to evaluate changes in these cytokines upon IL-23/IL-23R blockade. IL-33 has been shown to increase in crushed lungs from short-

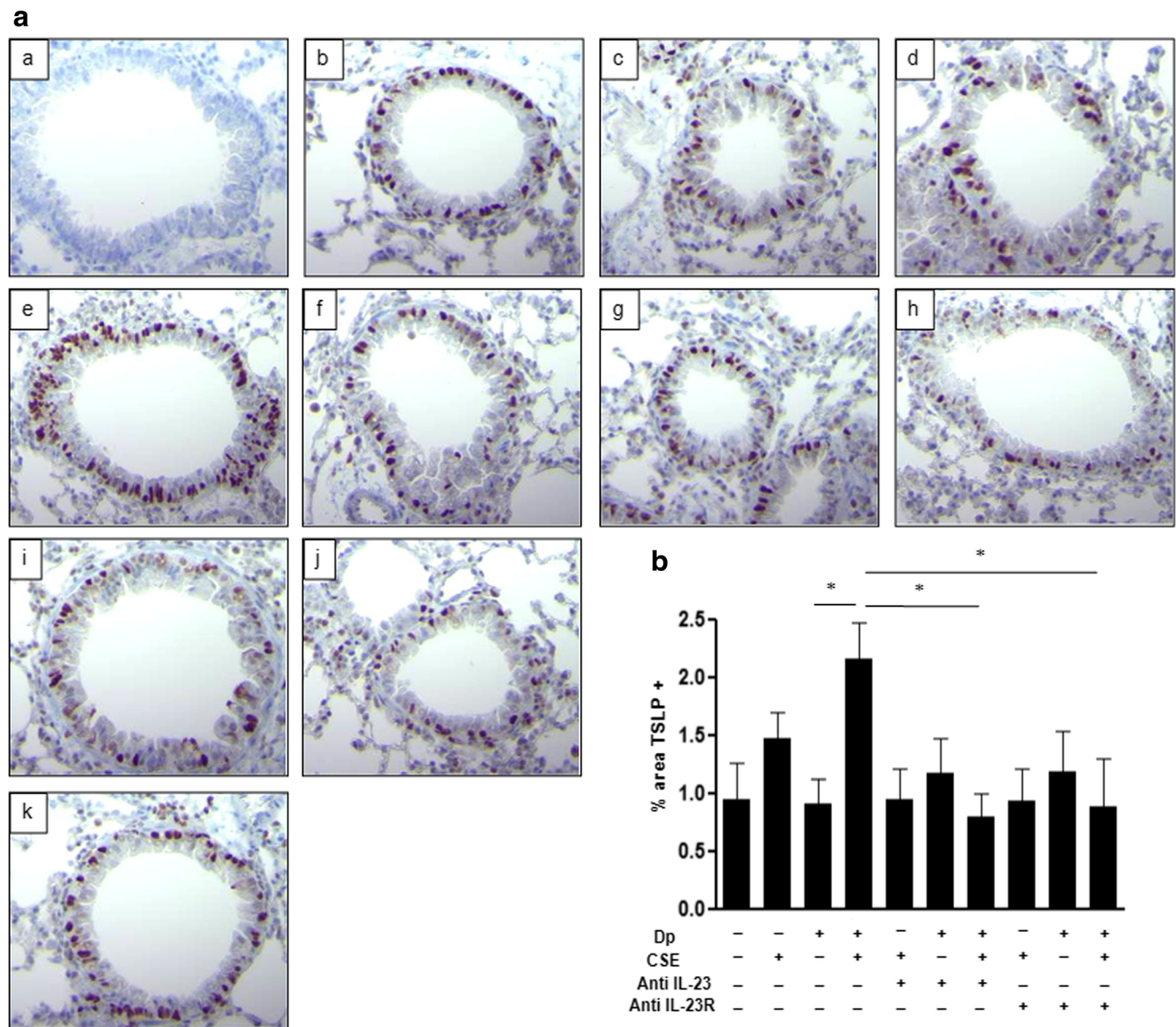


Fig. 7 The expression of TSLP from lung section in anti-IL-23 Ab or anti-IL-23R Ab-treated mice during sensitization period. After treatment of anti-IL-23 or IL-23R Ab in the sensitization period, mice were sacrificed 24 h after the last sensitization. **a** The expression of TSLP in lung section was detected using immunohistochemistry staining (IHC) (a:

isotype control, b: PBS, c: CSE, d: Dp, e: Dp/CSE, f: CSE + anti-IL-23 Ab, g: Dp + anti-IL-23 Ab, h: Dp/CSE + anti-IL-23 Ab, i: CSE + anti-IL-23R Ab, j: Dp + anti-IL-23R Ab, k: Dp/CSE + anti-IL-23R Ab, $\times 400$). **b** TSLP IHC quantification. $*p < 0.05$

term cigarette smoke/antigen exposed mice [8], suggesting that this innate cytokine may be important in the sensitization process. Furthermore, TSLP expression was not studied in the previous study, but we confirmed the changes in TSLP expression in this present study.

IL-23 is known to be closely related to Th17 cells and ILC3 [36, 37]. Therefore, we examined the changes in Th17 and ILC3 cells in this study. However, in our mouse model promoted by cigarette smoke at the time of sensitization, an increase in these cells was not observed (data not shown), indicating that these cells may not be major drivers of disease pathology in this model.

Meanwhile, there are a number of studies that have exposed cigarette smoke (CS) to asthmatic mice to determine the effects of smoking [38, 39]. However, some studies have shown that CS extract (CSE) also increases lung inflammation. It has been showed that CSE treatment also increases neutrophil and inflammatory cytokines [40]. In another asthma study, it was showed that administration of CSE to asthmatic mice exacerbated the development of asthma [41]. Therefore, although this method cannot perfectly recapitulate smoking in a mouse model, it is thought that the extract can be used as a component of a disease model in addition to the CS exposure method.

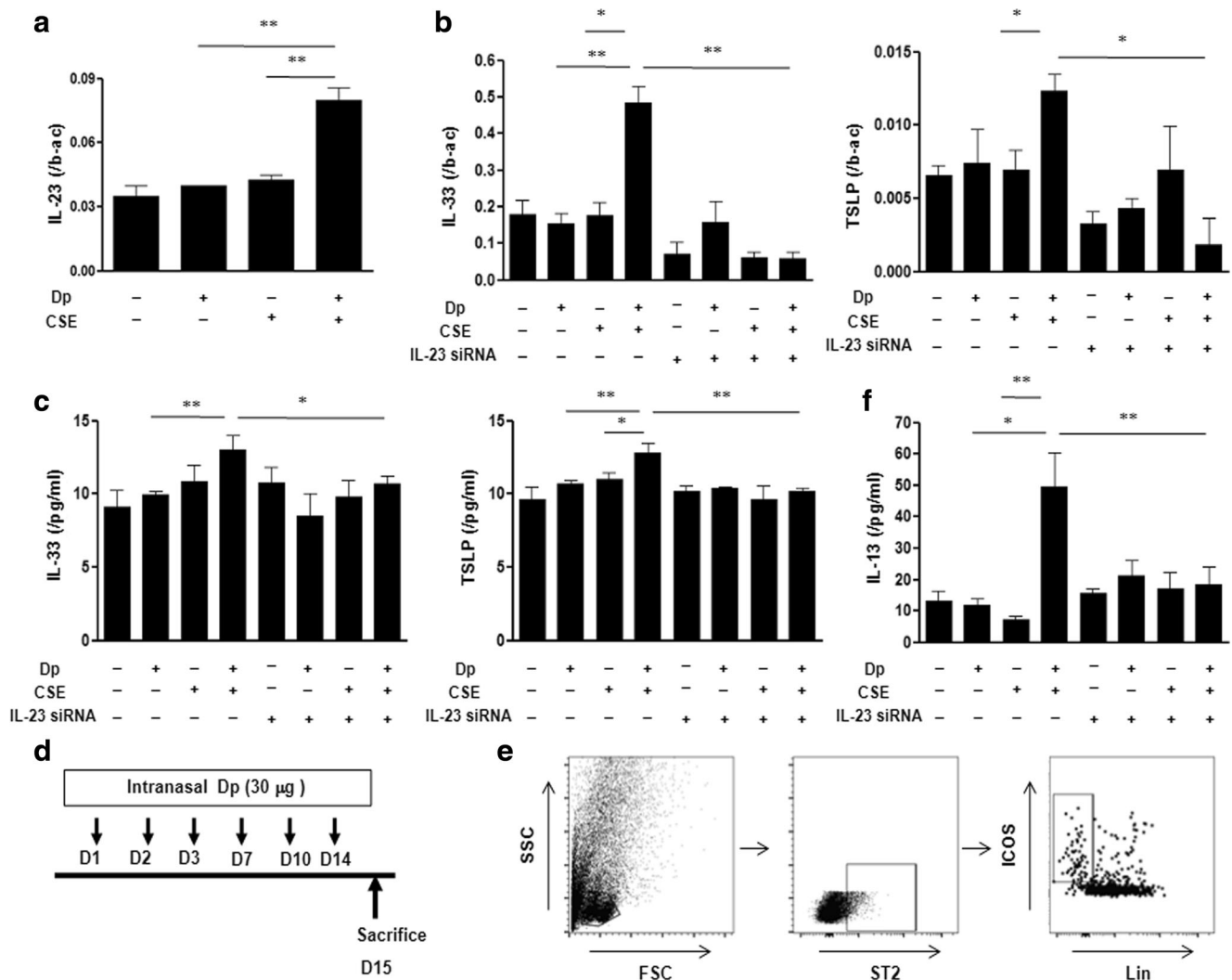


Fig. 8 Effects of anti-IL-23 Ab on the activation of ILC2 in Dp/CSE-stimulated MLE12 cells. **a–b** MLE-12 cells were detected the mRNA expression of IL-23, IL-33, and TSLP using real-time PCR. **c** The protein levels of IL-33 and TSLP from cytosol fraction using ELISA. **d** The protocol for sorting ILC2 from Dp-induced mice. **e** For isolation of ILC2, the population of ICOS+Lin- in ST2+ cells was sorted. For co-

cultivation, MLE-12 cells were treated IL-23p19 siRNA for 6 h. And these MLE-12 cells were stimulated by Dp (10 µg/ml) with or without CSE (2%/volume) for 5 h. After wash with PBS, were co-cultured with ILC2 for 48 h. **f** The level of IL-13 in ILC2 was determined using ELISA. * $p < 0.05$; ** $p < 0.01$

In contrast, some studies have reported that anti-asthmatic activities are observed when cigarette smoke and antigen are administered. Exposure of CS increases allergen sensitization as a consequence of increased GM-CSF levels. However, prolonged smoking exposure in the OVA challenge model showed an anti-inflammatory effect [42]. Subsequently, it was reported that eosinophilic inflammation was decreased in HDM-induced asthma mice only when mice were challenged [43]. Additionally, it was also shown that smoking exposure during the HDM challenge period did not further exacerbate the allergic phenotype [8]. Therefore, in the context of allergen challenge, smoking has an anti-inflammatory effect or no effect, but the underlying mechanism remains unclear and further studies are warranted. However, increased

Th2 immune responses and airway inflammation occur upon the simultaneous administration of OVA and CSE for 8 weeks [41]. The difference from the abovementioned study [42] was that OVA was administered with CSE throughout the whole period. Therefore, concurrent administration of allergen and cigarette smoke may be able to continually increase sensitization. We propose that differences occur according to variables such as the concurrent exposure of smoke and allergen or the duration of smoke or allergen exposure.

Recently, elevated IL-23 and IL-23R expression has been reported in pediatric asthma patients [44, 45]. Further, it was reported that young children may become more susceptible to allergic sensitization and ensuing asthma development [46]. Therefore, we speculate that IL-23 and IL-23R may be increased

by several irritants, such as cigarette smoke and antigen during childhood, which may promote the pathogenesis of asthma.

In our final experiment, we confirmed that changes induced by IL-23 in airway epithelial cells could lead to changes in the production of IL-13 by ILC2. However, further studies will be needed to determine the mechanistic details, whereby IL-33 and TSLP are regulated by IL-23 inhibition in Dp/CSE-stimulated airway epithelial cells.

One limitation of this study was that we were not able to establish a direct causal relationship of ILC2 because we could not experimentally remove ILC2 in this mouse model. However, many studies have suggested that ILC2 play a critical role in the development of asthma, i.e., allergen sensitization [47, 48]. In addition, an increase in IL-33 and TSLP levels was also suggested in a study of increased sensitization induced by smoking [8, 41]. There was no information about ILC2 provided in these studies, but considering the importance of this cell type, it is likely that ILC2 are involved. Furthermore, although the data are indirect, we used in vitro experiments to demonstrate a potential mechanism for our mouse model findings.

In conclusion, our findings suggest a critical role for IL-23 in the development of allergen sensitization promoted by cigarette smoke. As one potential mechanism, we suggest that IL-23/IL-23R signaling in airway epithelial cells may regulate the levels of innate cytokines, such as IL-33 and TSLP. Notably, IL-23R is known to play an important role in autoimmune diseases and psoriasis, whereas its role in asthma and allergic inflammation poorly defined. This present study suggests that the IL-23 pathway may provide a pharmacological target for the inhibition of cigarette smoke-driven asthma development.

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Compliance with ethical standards

All experiments were performed with the approval of the Institutional Animal Care and Use Committee of the Institute (IACUC) of Seoul National University. The approval number is SNU-160615-5-4.

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1 **Hyun Seung Lee et al.**

2

3 **Critical role of Interleukin-23 in development of asthma promoted by cigarette smoke**

4

5 **Supplementary Information**

6

7 **Supplementary Material &Methods**

8

9 **Preparation of Cigarette Smoke Extract (CSE)**

10 There search grade cigarettes (3R4F) were purchased from Kentucky Tobacco Research and
11 Development Center at the University of Kentucky (Lexington, KY, USA). CSE was prepared
12 following the method described previously[1]. We used modified cigarette smoke extract preparation
13 method. Briefly, one cigarette with a filter removed was fixed horizontally to be burned, and the main
14 stream of smoke was aspirated at a constant flow rate (one cigarette per 8 min) using variable flow
15 pump (C.B.S SCIENTIFIC, CA, USA). Ten cigarettes were passed through a solvent of 10 mL to
16 collect CSE, and this content was defined as 100% (one cigarette per 1 mL). This preparation was
17 sterilized through a 0.22-μm syringe filter and the CSE was prepared and stored at -80°C until use.

18

19 **ELISA assay, Immunohistochemistry (IHC) stain and immunofluorescence (IF) stain in lung tissue**

20 For detection of IL-23 expression in whole lung tissue, lungs were isolated and homogenized. Lysates
21 were then centrifuged at 15,000 g for 10 minutes, and supernatants were saved at -80 °C. The
22 production of IL-23 in crushed lung supernatant was measured by ELISA (Biolegend). And for
23 immunohistochemistry stain and immunofluorescence stain, the lung sections were incubated with as

each primary antibody. IL-23p19 (1:1000, Abcam), IL-23R antibody (1:1000, Abcam), IL-33 (1:50, R&D) and TSLP (1:4000, Abcam) antibody were used as primary antibodies. As isotype control, anti-rabbit or anti-goat IgG Ab was used. The slides mounted with DAPI for analysis. IF stain pictures were taken with a Leica fluorescence microscope and analyzed with a digital image program (LAS AF). IHC stain pictures were taken with a Nikon light microscope and analyzed with a digital image program (iSolution Lite). The slides were examined with the magnification of 400 x. Green means of IF stains were also quantified using Image J software. Average area % of IHC stains were quantified positive area with 4-5 fields/mice (n=3 mice) by Image J software after setting the thresholds.

Measurement of methacholine hyper-responsiveness and analysis of bronchoalveolar lavage fluid

One day after the last challenge with Dp, airway hyperresponsiveness (AHR) was measured with a barometric plethysmographic chamber (OCP 3000, Allmedicus, Anyang, Korea), which provides a noninvasive measure of airway responsiveness in mice. Mice were exposed to increasing doses of methacholine using an ultrasonic nebulizer (Pari, Starnberg, Germany) for 150 s at each concentration. AHR was calculated in enhanced pause (Penh). The formula used was as follows: $Penh = (T_e/RT - 1) \times PEF/PIF$, where T_e =expiration time (s), RT =relaxation time (s), PEF =peak expiratory flow rate (mL/s) and PIF =peak inspiratory flow rate (mL/s). And 24 hours after the assessment of airway hyperresponsiveness, mice were sacrificed and bronchoalveolar lavage fluid and lung tissue were obtained.

Histopathology

To evaluate and compare the severity and character of pathological changes in lung parenchyma, left lungs of mice were fixed in 10% neutral buffered formalin and embedded in paraffin, and 3-mm sections were stained with hematoxylin and eosin (H & E).

48

49 **Analysis of Th2 cells and type 2 innate lymphoid cells (ILC2) in lung tissues**

50 In the supernatants of crushed lungs, TSLP and IL-33 were measured using ELISA (R&D
51 Systems, Abingdon, UK) following the manufacturer's instructions. Type 2 innate lymphoid cells
52 (ILC2) in lung were determined by flow cytometry. After the preparation of single cell, the cells were
53 stimulated by PMA (100ng/ml), ionomycin (1ug/ml) and golgistop. Cells were stained with Percp-
54 cy5.5-conjugated anti-CD45 (eBioscience). For detection of ILC2s, Lineage-negative cells were gated
55 as cells not expressing CD3, CD4, CD8, CD11b, CD11c, CD19, F4/80, FcεRI and CD49b. And PE-
56 conjugated ST2, APC- conjugated CD25 also stained. For intracellular staining, cells were
57 permeabilized (Cytotfix/Cytoperm kit; BD Biosciences) and incubated with PE-cy7-conjugated anti-IL-
58 13 (eBioscience). For analysis of Th2 cells, cells were stained with Percp-cy5-conjugated anti-CD4
59 and PE-cy7-conjugated anti-IL-13. For the analysis of data using flow cytometry, each sample was read
60 500,000 cells on LSRII (BD Biosciences).

61

62 **Sorting of type 2 innate lymphoid cell (ILC2)**

63 Mice were treated with Dp (30ug/mouse) for 2 weeks. Lungs were harvested and for sorting of ILC2s,
64 Lineage-negative cells were gated as cells not expressing FITC- conjugated CD3, CD4, CD8, CD11b,
65 CD11c, CD19, F4/80, FcεRI and CD49b. And PE- conjugated ST2, APC- conjugated ICOS also
66 stained. Several Dp induced mice were pooled for sorting of ILC2s. FACS Aria II was used (BD
67 bioscience) for sorting of ILC2. Sorted lung ILC2 (1×10^4 /well) was expanded in vitro in 10% FBS
68 RPMI complete medium containing IL-2 (10ng/ml) and IL-33 (10ng/ml) for 7 days.

69

70 **Co-culture of MLE12 cells and ILC2 in the presence of transwell inserts**

71 For indirect co-cultivation of epithelial cell and ILC2, to prevent direct interaction between MLE12
 72 cells and ILC2 in the co-culture, 12-well plate with transwell inserts (pore size: 0.4 μ M) (BD
 73 Biosciences Corp., San Jose, CA, USA) were used to separate these two cells into two compartments.
 74 MLE12 cells were seeded in upper chamber in transwell (2×10^5 /well). After treating MLE12 cells to
 75 IL-23p19 siRNA 30 nM / well) or normal control (NC) siRNA using transfectamine (invitrogen) for 5h
 76 and the cell was washed, and low dose Dp (10 μ g / ml) with or without CSE (2%) for 5 h, the cell
 77 was washed with PBS. All siRNA was purchased from Santa Cruz Biotechnology. And ILC2 was
 78 cultured together in the presence of transwell inserts, in which MLE12 cells and ILC2 were placed in
 79 the upper and lower compartment. They were incubated for 48 h. The cell culture supernatant from
 80 ILC2 was collected and determined concentrations of cytokines using ELISA. The cells from MLE12
 81 were collected and determined expression of each gene within each sample was normalized against β -
 82 actin and expressed relative to the control sample using the formula $2^{-\Delta\Delta Ct}$, in which $\Delta\Delta Ct = (Ct$
 83 mRNA – Ct b-actin). For detect the level of IL-33 and TSLP in MLE12 cells, Nuclear/Cytosol
 84 Fractionation Kit (BioVision) was used.

85

86 **Supplementary Figures**

A

B

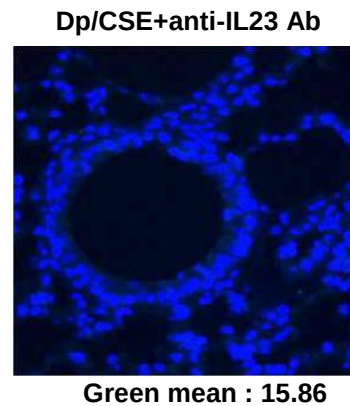
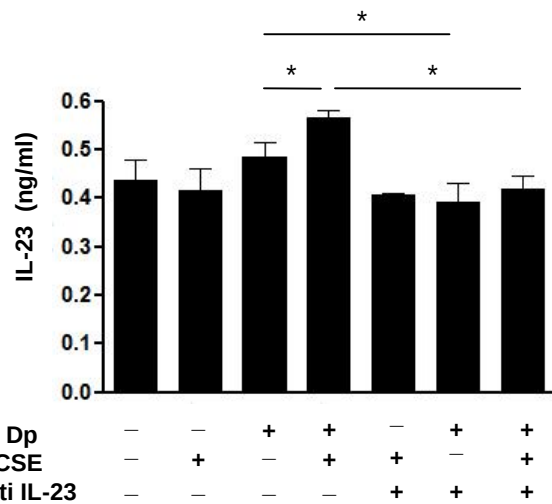


Fig. S1. The expression of IL-23 in anti IL-23Ab treated mice during sensitization period

(A) The protein level of IL-23 in crushed lung after the last sensitization was detected using ELISA. (B) The expression of IL-23 in lung after the last sensitization was detected using immunofluorescence staining (green). * $p < 0.05$.

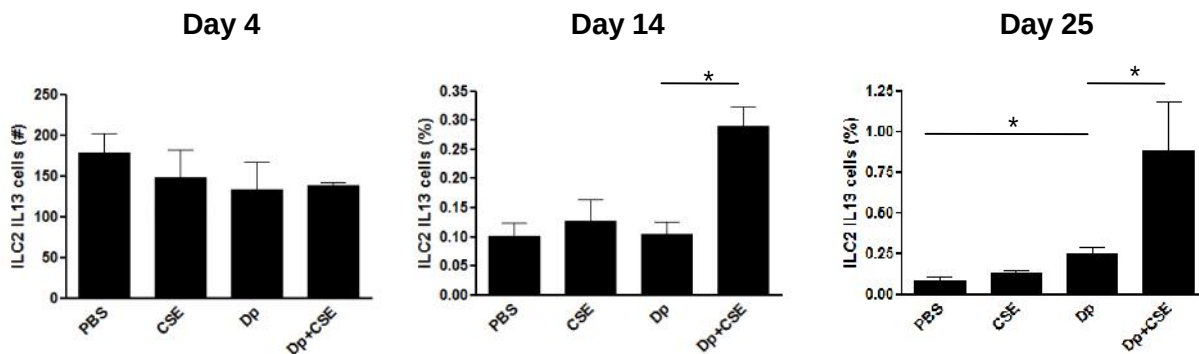


Fig. S2. The population of IL13+ILC2 in three time point.

IL13-produced type 2 innate lymphoid cells from lung were evaluated in Day 4, Day 14 and Day 25 in Dp with or without CSE treated mice during sensitization. * $p < 0.05$.

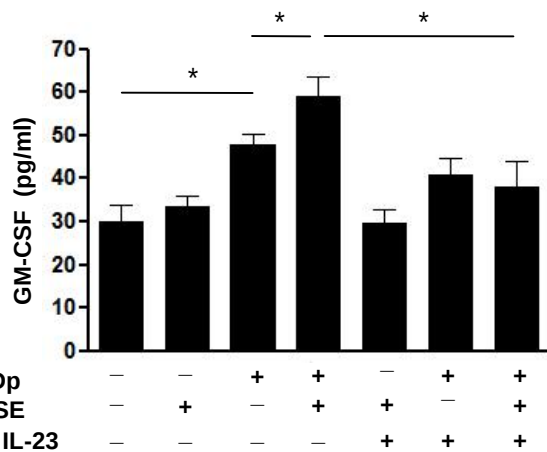


Fig. S3.The level of GM-CSF after the last sensitization

The levels of GM-CSF in the supernatants of crushed lungs were determined using ELISA. * $p < 0.05$.

Supplementary References

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