

IL-23 secreted by bronchial epithelial cells contributes allergic sensitization in asthma model

The role of IL-23 secreted by bronchial epithelial cells

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23 **Abstract**

24 **Background:** IL-23 has been postulated to be a critical mediator contributing to various inflammatory
25 diseases. *Dermatophagoides pteronyssinus* (Der p) is one of the most common inhalant allergens.
26 However, the role of IL-23 in Der p-induced mouse asthma model is not well understood, particularly
27 with regard to the development of allergic sensitization in the airways.

28 **Objective:** To evaluate roles of IL-23 in Der p-sensitization and asthma development.

29 **Methods:** BALB/c mice were repeatedly administered with Der p intra-nasally to develop Der p-
30 allergic sensitization and asthma. After Der p local administration, changes in IL-23 expression were
31 examined in lung tissues and primary epithelial cells. Anti-IL-23p19 antibody was given during the
32 Der p sensitization period, and its effects were examined. Effects of anti-IL-23p19 antibody at
33 bronchial epithelial levels were also examined in vitro.

34 **Results:** The expression of IL-23 at bronchial epithelial layers was increased after Der p local
35 administration in mouse. In Der p-induced mouse models, anti-IL-23p19 antibody treatment during
36 allergen sensitization significantly diminished Der p-allergic sensitization and several features of
37 allergic asthma including the production of Th2 cytokines and the population of ILC2 in lungs. The
38 activation of dendritic cells in lung-draining lymph nodes was also reduced by anti-IL-23 treatment. In
39 MLE-12 cells, IL-23 blockade prevented cytokine responses to Der p stimulation, such as IL-1 α , GM-
40 CSF, IL-33, and also bone marrow-derived DCs activation.

41 **Conclusion:** IL-23 is another important bronchial epithelial cell-driven cytokine which may contribute
42 to the development of house dust mite allergic sensitization and asthma.

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44 **Keywords:** IL-23, bronchial epithelial cell, *Dermatophagoides pteronyssinus*

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47 **Introduction**

48 Allergic asthma is characterized by Th2-predominant immune responses to inhaled allergens
49 such as house dust mites (25). Bronchial epithelium is the primary site for tissue surveillance, and
50 responds to inhaled stimuli including pollutants, pathogens, and allergens (10, 36). Thus, the roles of
51 bronchial epithelium in the pathogenesis of allergic sensitization and asthma are considered significant
52 (11).

53 IL-23 is a pro-inflammatory cytokine identified as a member of the IL-12 family. IL-23 is
54 composed of p40, a subunit of IL-12, and p19, which is a unique subunit for IL-23 function (28). The
55 main cellular source of IL-23 is reported to be dendritic cells (DC) (18). The biological functions of
56 IL-23 have been relatively well known for the capability to enhance Th17 cell functions (1, 28), and
57 thus have been implicated in various autoimmune disorders and infectious conditions (14, 16). In
58 asthma, several studies have reported the roles of IL-23 as follows: IL-23 production is increased in
59 ovalbumin (OVA)-induced murine asthma models (19) and IL-23 promotes the development and
60 maintenance of Th17 cells and also the recruitment of eosinophils and neutrophils to the airways (30).
61 However, these studies have examined the roles of IL-23 in regard to DC, T cells, and granulocytes,
62 but not to bronchial epithelium. Considering the functional expression of IL23p19 in intestinal
63 epithelium (24) and keratinocytes (22), we hypothesized that IL-23 could also be expressed in
64 bronchial epithelium, and be involved in bronchial epithelial responses to inhaled allergen, which has,
65 to our knowledge, not been previously reported. Furthermore, we postulated that IL-23 could be
66 involved in the development of allergic sensitization and subsequent asthma.

67 In this study, we aimed to examine the expression of IL-23 at bronchial epithelium after
68 allergen exposure, and investigate whether IL-23 plays pivotal roles in the development of allergic
69 sensitization and asthma.

70

71 **Materials and Methods**

72 **Animals**

73 Six weeks old female BALB/C mice (18-20 g) were purchased from SLC Inc. (Hamamatsu,
74 Kotoh-cho, Japan). All experiments were performed with the approval of the Institutional Animal Care
75 and Use Committee of the Institute (IACUC) of Laboratory Animal Resources at Seoul National
76 University (SNU-150604-1).

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78 **Expression of IL-23 in lung tissue and bronchial epithelium using western blot assay,** 79 **immunohistochemistry stain and immunofluorescence stain**

80 For detection of IL-23 expression in whole lung tissue, lungs were isolated and homogenized.
81 Lysates were then centrifuged at 15,000 g for 10 minutes, and supernatants were saved at -80 °C.
82 Primary antibodies used were: IL-23p19 (1:1000, Abcam) and β -actin (1:5000, Abcam) for western
83 blot assay. The production of IL-23 in crushed lung supernatant was measured by ELISA (Biolegend).
84 And for immunohistochemistry stain (1:2000) and immunofluorescence stain (1:1000), the lung
85 sections were incubated with IL-23p19 antibody (Abcam) as primary antibody. As isotype control,
86 anti-rabbit IgG Ab was used. After mounting on slides with DAPI, the immunofluorescent staining was
87 analyzed using Leica microscope.

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89 **Expression of IL-23 and IL-23R in primary tracheal epithelial cell line**

90 The primary tracheal epithelial cell line were purchased from Cell Biologics (cat no. C57-6033,
91 Chicago, USA). These cells (passage 2) were cultured with epithelial cell medium (Cell Biologics) in
92 pre-coated culture plate and exposed for 24 h to Der p (80 μ g) or Medium as a control. The expression
93 of IL-23p19 (1:200, Abcam) of adherent cells was detected in 24-well pre-coated coverslip in plate

94 using immunofluorescence staining. As isotype control, 488-conjugated anti-rabbit IgG Ab was used.
95 The expression of Epcam (PE-conjugated, eBioscience) and IL-23R (PE-conjugated, R&D) was
96 detected using flow cytometry. As isotype control, PE-conjugated anti-rat IgG1 Ab was used.

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98 **Development of allergic asthma models and anti IL-23 or anti IL-1 α antibody treatment**

99 Dermatophagoides pteronyssinus (Der p) extract was provided from Yonsei university hospital
100 (Seoul, Korea) (34). Goat anti-mouse IL-23p19 Ab (2 μ g) and Armenian hamster anti-mouse IL-1 α Ab
101 (100 μ g) was used to block IL-23 or IL-1 α bioactivity in Der p (50 μ g) sensitized mice. Goat or
102 Armenian hamster IgG Ab was also treated in Der p sensitized mice as control. This dose of anti IL-
103 23p19 Ab has been shown to successfully neutralize IL-23 or IL-1 α activity in vivo (3, 6). Der p was
104 resuspended in phosphate-buffered saline (PBS) and mice were instilled by intranasal injection (Fig.
105 3A).

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107 **Measurement of methacholine hyper-responsiveness and analysis of bronchoalveolar lavage fluid** 108 **and serum**

109 One day after the last challenge with Der p, airway hyperresponsiveness (AHR) was measured
110 with a barometric plethysmographic chamber (OCP 3000, Allmedicus, Anyang, Korea) and 24 hours
111 after the assessment of airway hyperresponsiveness, mice were sacrificed and bronchoalveolar lavage
112 fluid and lung tissue were obtained. BAL fluid inflammatory cells were obtained as previously
113 described (12). The level of cytokines in BAL fluid was measured using commercially available
114 ELISA Kit (R&D Systems Inc., Minneapolis, MN, USA) as manufacturer's instruction. Serum Der p-
115 specific IgG1 was measured by using ELISA.

116

117 **Histopathology**

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

121

122 **Analysis of cytokines and type 2 innate lymphoid cells (ILC2) in lung tissues**

123 In the supernatants of crushed lungs, IL-1 α and IL-33 were measured using ELISA (R&D
124 Systems, Abingdon, UK) following the manufacturer's instructions. Type 2 innate lymphoid cells
125 (ILC2) in lung were determined by flow cytometry. Cells were stained with Percp-cy5.5-conjugated
126 anti-CD45 (eBioscience). For detection of ILC2s, Lineage-negative cells were gated as cells not
127 expressing CD3, CD4, CD8, CD11b, CD11c, CD19, F4/80, Fc ϵ RI and CD49b. And PE- conjugated
128 ST2, APC- conjugated CD25 also stained. For intracellular staining, cells were permeabilized
129 (Cytofix/Cytoperm kit; BD Biosciences) and incubated with PE-cy7-conjugated anti-IL-13
130 (eBioscience). For analysis of Th2 cells, cells were stained with Percp-cy5-conjugated anti-CD4 and
131 PE-cy7-conjugated anti-IL-13.

132

133 **Evaluation of DC activation in Lung Draining Lymph Node (LDLN)**

134 On day 0, 1, and 2, the immunized mice were treated with anti IL-23p19 Ab or isotype Ab.
135 Mice were sacrificed 24 hour after last sensitization with Der p. The LDLNs were removed and
136 processed. The percentage of DCs was determined by flow cytometry as the fraction of MHCII⁺ or
137 CD86⁺ in CD11c⁺ cells.

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139 **Measurement of cytokine levels in lung epithelial cells**

140 MLE12 cell line, a mouse lung epithelial cell line, was purchased from the ATCC (Manassas,
141 VA, USA). The cells were grown in DMEM/F12 medium with 2% FBS in a humidified atmosphere at

142 37°C with 5% CO₂. After exposing MLE12 cells to Der p (80 µg / ml) and anti IL-23p19 Ab (0.05
143 µg/ml, R&D systems), anti IL-23R Ab (0.05 µg/ml, R&D systems), or recombinant mouse IL-23 (0.01
144 µg/ml, ebioscience) for 24 h, the cell culture supernatants were collected and determined
145 concentrations of cytokines using ELISA. Expression of each gene within each sample was normalized
146 against β-actin and expressed relative to the control sample using the formula $2^{-\Delta\Delta Ct}$, in which
147 $\Delta\Delta Ct = (Ct \text{ mRNA} - Ct \text{ b-actin})$.

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149 **Generation of bone marrow-derived DCs (BMDCs)**

150 BMDCs were differentiated from bone marrow cells by methods previously reported (7, 29) .
151 After 7 days of culture, DC-specific markers (CD11c⁺) as determined by flow cytometry.

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153 **Coculture of MLE12 cells and BMDCs in the presence of transwell inserts**

154 For indirect co-cultivation of epithelial cell and dendritic cell, to prevent direct interaction
155 between MLE12 cells and BMDCs in the coculture, 12-well plate with transwell inserts (pore size: 0.4
156 mM) (BD Biosciences Corp., San Jose, CA, USA) were used to separate these two cells into two
157 compartments. MLE12 cells were seeded in upper chamber in transwell. After treating MLE12 cells to
158 IL-23p19 siRNA 30 nM / well) or IL-23R siRNA (30 nM / well) or normal control (NC) siRNA using
159 transfectamine (invitrogen) and Der p (80 µg / ml) for 24 h, or treating MLE12 cells to recombinant
160 mouse IL-23 (0.01 µg/ml) for 24 h, the cell was washed with PBS. All siRNA was purchased from
161 Santa Cruz Biotechnology. And BMDCs were cultured together in the presence of transwell inserts, in
162 which MLE12 cells and BMDCs were placed in the upper and lower compartment. They were
163 incubated for 24 h. BMDCs were then incubated with the following anti-mouse antibodies: APC/cy7-
164 conjugated anti-CD11c, APC-conjugated anti-MHCII and PE-conjugated anti-CD86 (all from
165 eBioscience).

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Analysis of data using flow cytometry

Samples were analyzed on LSRII (BD Biosciences, San Jose, CA) 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 Prizm 4.01 (GraphPad Software, La Jolla, CA, USA). *P*-values < 0.05 were considered significant.

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Results

The expression of IL-23 was increased from lung epithelium in Der p-exposed mice during sensitization period and in Der p-stimulated primary ECs

First, we examined the expression of IL-23 in mouse lung tissues after the end of the Der p sensitization period. In ELISA from crushed lung sup and Western blot assay (n=3), the expression of IL-23 was found to be increased in whole lung tissues in Der p-exposed mice compared to control mice. (Fig. 1B, C). In immunohistochemistry staining and immunofluorescence staining of lung sections, the expression of IL-23 was found to be increased by Der p inhalational exposure (Fig. 1D, E). It was confirmed that there was no expression in 2nd isotype control Ab staining, as negative control. In the above experiments, IL-23 was found to be expressed at bronchial epithelial layers in mice. Therefore, we sought to confirm that expression in primary mouse tracheal epithelial cell. To find out the expression, we used primary mouse tracheal epithelial cell line. Most of these cells were expressed Epcam (Fig. 1F). After these cells were grown on coverslips, it was performed immunofluorescence staining. In immunofluorescence staining, the expression of IL-23 was detected in non-stimulated tracheal epithelial cell line. And, this expression was increased in Der p-stimulated tracheal epithelial cell line (Fig. 1G). In addition, the expression of IL-23R was also detected in this cell. As a result, the surface expression of IL-23R was increased in Der p-stimulated tracheal epithelial cell line (Fig. 1H).

Airway inflammation and AHR decreased in anti-IL-23 Ab treated mice during Der p sensitization

To examine the role of IL-23 during sensitization in Der p induced asthma model, an experiment was conducted by systemic administration of anti-IL-23p19 Ab (Fig. 2A). The numbers of

214 inflammatory cells from BALF, including eosinophils, were also significantly reduced in anti-IL-23
215 Ab treated mice compared to control (Fig. 2B). The recruitment of inflammatory cells into the lungs of
216 mice was also investigated by histopathological studies. The infiltration of inflammatory cells,
217 including eosinophils and lymphocytes, was found to be significantly reduced by anti-IL-23 Ab
218 treatment (Fig. 2C, a-c). In methacholine challenge tests, AHR was significantly reduced in anti-IL-23
219 Ab-treated mice compared to isotype Ab-treated mice among those that developed Der p allergic
220 asthma (Fig. 2D).

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222 **Expressions of cytokines and IL-13 producing ILC2 cells decreased in anti-IL-23-treated mice** 223 **during Der p sensitization**

224 In BALF, the levels of IL-4, IL-5, IL-17A and Der p-specific IgG1 levels were found to be
225 significantly reduced by anti-IL-23 Ab treatment (Fig. 3A-D). Recent studies demonstrated that HDM
226 could affect ILC2-dependent production of Th2 cytokines (8). In addition, the roles of ILC2 have been
227 suggested in the development of allergic asthma (8). Therefore, we explored the effects of IL-23
228 blockade on ILC2 cells from lung after the allergen challenge, and found that treatment of anti-IL-23 to
229 Der p sensitized mice reduced the population of IL13-producing ILC2 cells in Lin-⁺ST2⁺CD25⁺ cells
230 (Fig. 3E, F).

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232 **The effect of IL-23 on innate pro-Th2 cytokines and DC activation in lung draining lymph node**

233 To explain the mechanisms of ILC2 reduction by IL-23 blockade during the Der p sensitization
234 period, we proceeded to measure possible changes of innate pro-Th2 cytokines, including IL-1 α and
235 IL-33, in whole lung tissues using ELISA (Fig. 4A). The levels of IL-1 α and IL-33 from whole lung
236 tissues were significantly reduced by anti-IL-23 Ab treatment (Fig. 4B, C). IL-1 α and IL-33 are known
237 to be important in mediating DC activation (36); thus, we examined whether the activation of DC from

238 LDLN was changed by anti-IL-23 Ab. It was found that the activation of DC was also significantly
239 reduced by anti-IL-23 Ab treatment (Fig. 4D-F).

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241 **AHR, eosinophils and IL-13 producing ILC2 cells decreased in anti-IL-1 α Ab treated mice**
242 **during Der p sensitization**

243 We evaluated that the levels of IL-1 α and IL-33 from whole lung tissues were significantly
244 reduced by anti-IL-23 Ab treatment. A recent study demonstrated that IL-1 α secreted by airway
245 epithelial cells controlled allergic sensitization to inhaled house dust mite via the epithelial release of
246 GM-CSF and IL-33 (36). Therefore, to examine the role of IL-1 α during sensitization in Der p induced
247 asthma model, an experiment was conducted by systemic administration of anti- IL-1 α Ab. AHR, the
248 numbers of eosinophils from BALF were significantly reduced in IL-1 α Ab treated mice compared to
249 control (Fig. 5A-B). In addition, the population of IL13-producing CD4⁺ T cells (Fig. 5C-D) and the
250 population of IL13-producing ILC2 cells also reduced in IL-1 α Ab treated mice compared to control
251 (Fig. 5E-F).

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253 **Blockade of IL-23 inhibited Der p-induced protein production of innate pro-Th2 cytokines in**
254 **MLE12 cells**

255 To support the experimental findings above, we examined cellular responses of bronchial
256 epithelial cell line, MLE12. First, it was confirmed that the expression of IL-23p19 and IL-23 receptor
257 (IL-23R) was increased by Der p exposure in vitro (Fig. 6A-C). In addition, the levels of IL-1 α and
258 GM-CSF and the expression of IL-33 were increased in Der p-stimulated MLE12 cells. However, the
259 levels of IL-1 α , GM-CSF, and IL-33 were significantly reduced by treatment with anti-IL-23 antibody;
260 thus, we hypothesized that IL-23 could have an autocrine mode of action on bronchial epithelial cells.

Findings showed the levels of IL-1 α , GM-CSF, and the expression of IL-33 in Der p-stimulated MLE12 cells were also significantly reduced by anti-IL-23R blockade, as well as anti-IL-23 blockade. But, these cytokines were significantly increased by recombinant IL-23 treatment in MLE12 cells (Fig. 6D-F). These findings suggested a potential autocrine pathway of IL-23 function at bronchial epithelial cells, in response to Der p.

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Blockade of IL-23 in Der p-stimulated epithelial cells inhibited BMDCs activation

Next, we determined whether the change of Der p stimulated lung epithelial cells by inhibition of IL-23 could affect the activation of BMDCs. After co-cultivation of Der p plus each siRNA or recombinant IL-23 pre-stimulated MLE12 cells with BMDCs for 24 h on indirect system. And the population of CD11c⁺MHCII⁺ cells and CD11c⁺CD86⁺ cells were analyzed from BMDCs. In case of Der p plus IL-23 siRNA or IL-23R siRNA-pretreated MLE12 cells, the population of MHCII⁺ and CD86⁺ in CD11c⁺ cells were significantly reduced when co-cultured with BMDCs compared to Der p-pretreated MLE12 cells co-cultured with BMDCs. However, in case of co-cultivation of recombinant IL-23 pre-stimulated MLE12 cells with BMDCs, MHCII⁺ and CD86⁺ in CD11c⁺ cells were significantly increased compared to control well (Fig. 6G, H).

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287 **Discussion**

288 IL-23 has been suggested to play pathogenic roles in various immunologic disorders such as
289 experimental autoimmune encephalomyelitis (15), collagen-induced arthritis (37), and inflammatory
290 bowel disease (33). In asthma, roles of IL-23 were mostly investigated in OVA-induced asthma models.
291 In these experiments, eosinophilic airway inflammation, cytokine synthesis including Th2 cytokines
292 and IL-17 were significantly suppressed by IL-23 inhibition (19). Moreover, IL-23 signaling has been
293 shown to regulate allergic asthma through the modulation of Th2 cell differentiation as well as Th17
294 cell differentiation (35). These results are supported by clinical studies; serum IL-23 levels were
295 significantly related to lower lung functions in 85 young patients with asthma (5).

296 Recently, the role of IL-23 has been shown to be important during early phase in
297 epicutaneously-sensitized asthma model (17, 27). In these studies, it was demonstrated that IL-23 plays
298 an essential role during sensitization in the development of allergic phenotypes.

299 House dust mite sensitization is essential to induction of the adaptive immune response, and the
300 airway epithelial cells in the process has been known to play many roles in immune response induction
301 (13). However, the mechanisms by which Der p exposure finally leads to allergic sensitization and
302 asthma are not clear. In this regard, the present study suggests IL-23 to be one of the important
303 mediators in pro-allergic pathways in response to Der p exposure of bronchial epithelium.

304 The cellular source of IL-23 appears to be various in the literature. For asthma, activated DCs
305 and macrophages have been reported as the main cellular sources (18). However, studies on
306 gastrointestinal or cutaneous inflammatory conditions and oral lichen planus have suggested their
307 epithelial cells to be the sources of IL-23 production (20, 23). Moreover, it has been shown that IL-
308 23p19 constitutively expressed by normal human skin keratinocytes. It has also been reported that

309 keratinocyte-derived IL-23 amplifies cytokines from memory T cells (32). However, none of previous
310 studies have focused on the expression and functions of IL-23 at bronchial epithelial cell levels, and
311 furthermore have examined its responses to house dust mite allergens. Therefore, we investigated the
312 secretion of IL-23 and the role of IL-23 in bronchial epithelial cells. Here, we found for the first time
313 that IL-23 is increased at bronchial epithelium in response to Der p exposure. Our findings suggest
314 potential functions of bronchial epithelial cell-driven IL-23 in asthma pathogenesis.

315 Therefore, we inhibited IL-23 only during sensitization process-induced murine asthma model
316 by house dust mite. Findings showed these asthmatic phenotypes, Th2 cytokines and Der p-specific
317 IgG1 after challenge were reduced by inhibition of IL-23 during the sensitization period.

318 ILC2 is considered to play key roles in initiating and maintaining Th2 responses, particularly
319 driven by bronchial epithelial responses to stimuli including allergens (2, 9). In house dust mite allergic
320 asthma models, ILC2 were found to be indispensable in the development of asthma (26). Here, we also
321 found that lung ILC2 population significantly increased by Der p exposure. In present study,
322 interestingly, we also found that the ILC2 was significantly reduced by IL-23 inhibition. As a
323 mechanism of this result, the expression of IL-33 from lung tissues and bronchial epithelial cell lines in
324 response to Der p stimulation were also reduced by anti-IL-23 treatment. Therefore, our findings could
325 suggest that IL-23 is another epithelial-driven key mediator for inducing ILC2 responses to house dust
326 mite allergens, possibly also with the ability to regulate IL-33 responses.

327 The innate cytokines such as GM-CSF and IL-33 secreted in airway epithelial cells play a role
328 in induction of the Th2-biased airway inflammation by the recruitment and activation of DC (4, 21).
329 Thus, it could be speculated that the recruitment and activation of DC by GM-CSF or IL-33 secretion
330 in airway epithelial cells serve as important mechanisms to induce Th2 immune responses. In the
331 present study, we found that DC activation from LDLN was significantly reduced by inhibiting IL-23
332 after the Der p sensitization process. Therefore, we speculated that increasing expression of IL-23 in

333 Der p sensitized airway epithelial cells may be one of the factors in the activation DC and ILC2 cells.
334 To test this, we used the mouse bronchial epithelial cell line MLE-12 cell in the experiment. Findings
335 confirmed the secretion of GM-CSF as well as an increasing expression of IL-33 reduced by the
336 inhibition of IL-23 in Der p-stimulated MLE-12 cells.

337 It is still unclear whether IL-23 secreted by bronchial epithelial cells regulates IL-33 and GM-
338 CSF directly or at the upstream. A recent study demonstrated that IL-1 α secreted by airway epithelial
339 cells controlled allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF
340 and IL-33 (36). Therefore, we also detected the level of IL-1 α in lung tissue and MLE-12 cells. As a
341 result, interestingly, the level of IL-1 α was reduced in Der p with anti-IL-23 Ab-treated mice after
342 sensitization as well as in Der p with anti-IL-23 Ab stimulated MLE-12 cells. In addition, we
343 confirmed that several features including AHR, the number of eosinophils and Th2 cytokine after
344 challenge were reduced by inhibition of IL-1 α during the sensitization period in mouse model.

345 This result is indicative of the autocrine effect of IL-23 in bronchial epithelial cells.
346 Furthermore, clinical study has shown that the expression and polymorphism of IL-23R are related to
347 asthma (31). However, the expression of IL-23R in airway epithelial cells has not been reported
348 previously. We observed that surface expression of IL-23R was also increased in Der p stimulated
349 mouse primary tracheal epithelial. And, we evaluated the effect of IL-23R and recombinant IL-23 in
350 MLE-12 cells. We found that these innate cytokines were also reduced in anti-IL-23R Ab-treated Der
351 p-stimulated MLE-12 cells. In contrast, these innate cytokines significantly increased in recombinant
352 IL-23-treated MLE-12 cells. Collectively, our findings suggest that IL-23 may regulate these innate
353 cytokines by autocrine effect in MLE-12 cells.

354 In the final experiment, in order to prove that this reduction in the activation of dendritic cell is
355 due to the regulation of soluble mediators by IL-23 in airway epithelial cells, we confirmed the
356 reduction of DC activation after incubation with Der p and IL-23 siRNA or IL-23R siRNA-pretreated

357 MLE-12 cells.

358 The regulation of innate cytokines such as IL-1 α and GM-CSF and IL-33 secretion by IL-23 in
359 airway epithelial cells is not known yet. Therefore, further experiments are required to clarify this
360 mechanism.

361 In conclusions, our findings suggest pivotal roles of IL-23 in the development of Der p-allergic
362 sensitization and subsequent asthma. As one of mechanism, we suggest that epithelial cell-driven IL-23
363 may regulate innate cytokines such as IL-1 α and IL-33.

364

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484

485 **Figure legends**

486 **Figure 1. The expression of IL-23 in lung and primary tracheal epithelial cell line**

487 Experimental protocol of the study (A). The expression of IL-23 from lung was detected after
488 the last sensitization. The expression of IL-23 in lung from PBS and Der p mice was detected using
489 ELISA and western blotting (B, C). The expression of IL-23 in lung from PBS and Der p mice was
490 detected using immunohistochemistry staining (D) and immunofluorescence staining (green, E). Green
491 mean in immunofluorescence staining was analyzed using Image J program. The image of mouse
492 primary tracheal epithelial cell line and the expression of Epcam was detected using flow cytometry
493 (F). The expression of IL-23 (green) in primary tracheal epithelial cell line was detected using
494 immunofluorescence staining (G). The surface expression of IL-23R in primary tracheal epithelial cell
495 line was detected using flow cytometry (H). * $p < 0.05$

496

497 **Figure 2. The evaluation of asthmatic phenotypes in anti IL-23 Ab treated mice during** 498 **sensitization period.**

499 Experimental protocol for asthma model of study (A). The number of inflammatory cells
500 including eosinophils in BALF (B). Lung histology after the last challenge (C; a: PBS, b: Der p, c: Der p
501 + anti IL-23 Ab, H&E stain, $\times 100$). Methacholine hyperresponsiveness was measured 24 h after the last
502 challenge (D). * $p < 0.05$, ** $p < 0.01$.

503

504 **Figure 3. The expression of cytokines, Der p specific IgG1, the population of ILC2 after** 505 **challenge in anti IL-23 Ab treated mice during sensitization**

BALF was collected at 48 h after the last challenge. Expressions of IL-4, IL-5 and IL-17A from BALF after the last challenge in anti IL-23 Ab treated mice during sensitization (A-C). Serum Der p-specific IgG1 (D) was evaluated after the last challenge in anti IL-23 Ab treated mice during sensitization. IL13-produced type 2 innate lymphoid cells from lung (E, F) were evaluated after the last challenge in anti IL-23 Ab treated mice during sensitization. * $p < 0.05$, ** $p < 0.01$.

511

Figure 4. The level of innate pro-Th2 cytokines in crushed lung and the numbers of MHCII+ or CD86+ in CD11c+DCs after the last sensitization

Experimental protocol of the study (A). After treatment of anti IL-23 Ab in the sensitization period, mice were sacrificed 24 hrs after the last sensitization. The levels of IL-1 α and IL-33 in the supernatants of crushed lungs were determined using ELISA (B, C). After sacrifice, lung draining lymph nodes were isolated from mice. The numbers of MHCII+ cells (D, E) or CD86+ cells (F) in CD11c+ DCs were determined using flow cytometry. * $p < 0.05$, ** $p < 0.01$.

519

Figure 5. The evaluation of asthmatic phenotypes in anti IL-1 α Ab treated mice during sensitization period.

Methacholine hyperresponsiveness was measured 24 h after the last challenge (A). The number of eosinophils in BALF (B). IL13-produced CD4+ T cells (C, D) and IL13-produced type 2 innate lymphoid cells from lung (E, F) were evaluated after the last challenge in anti IL-1 α Ab treated mice during sensitization. * $p < 0.05$, ** $p < 0.01$.

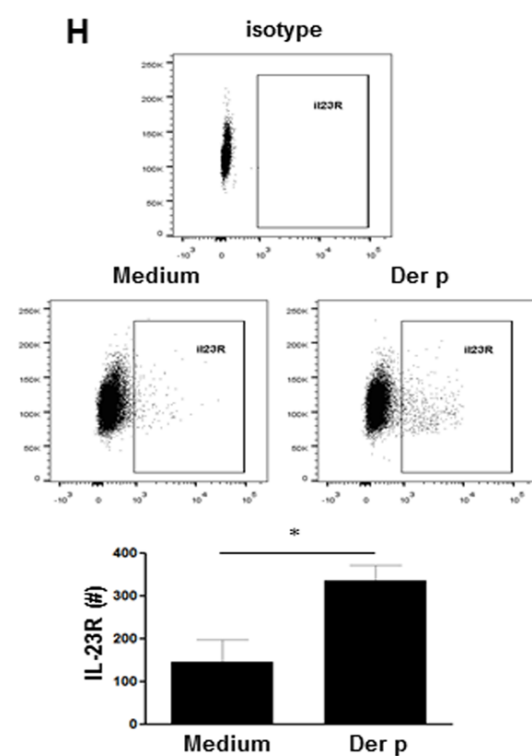
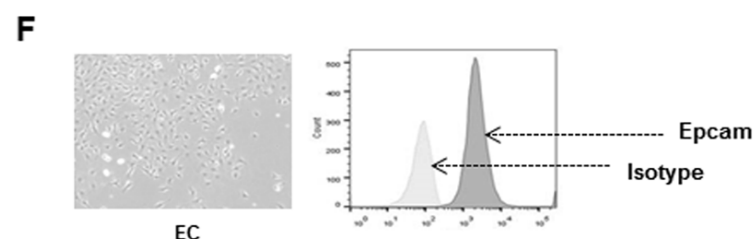
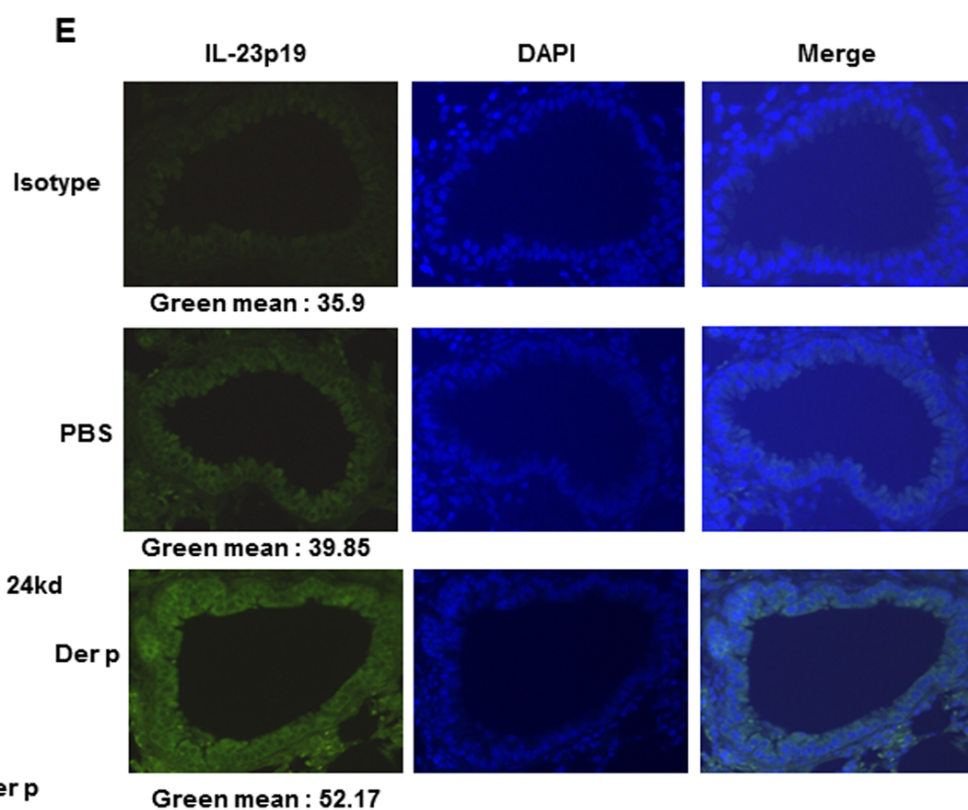
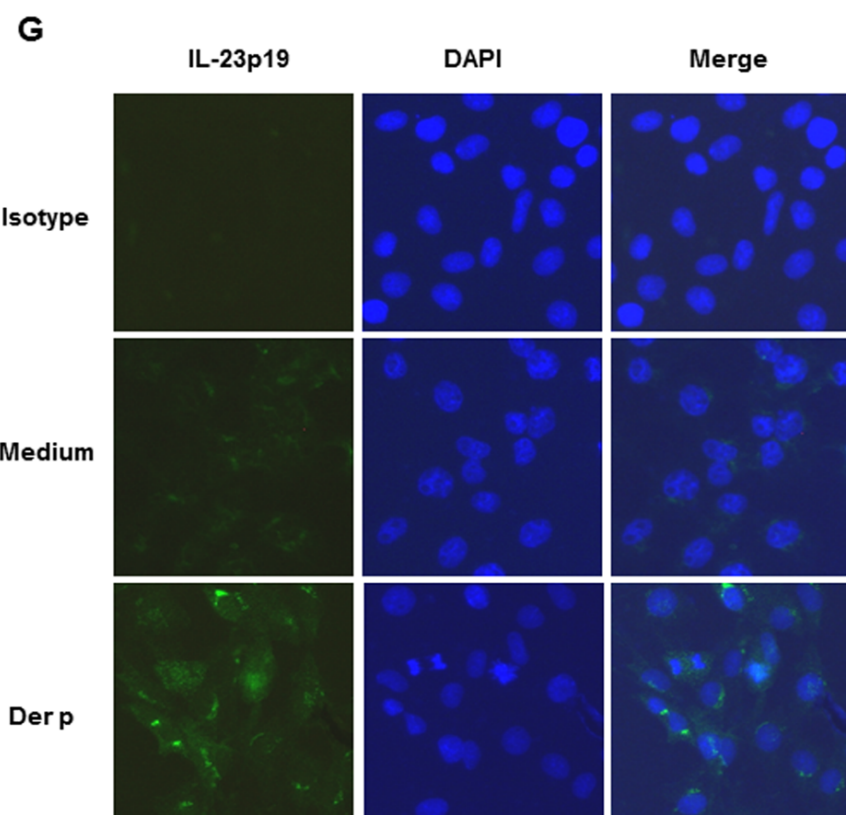
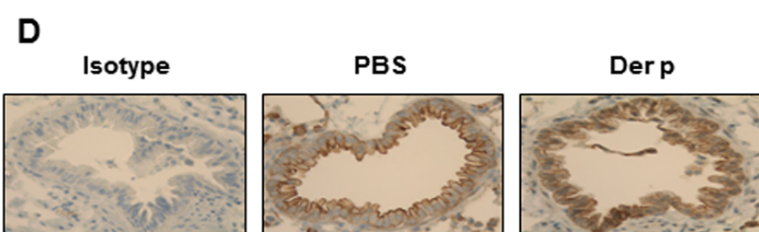
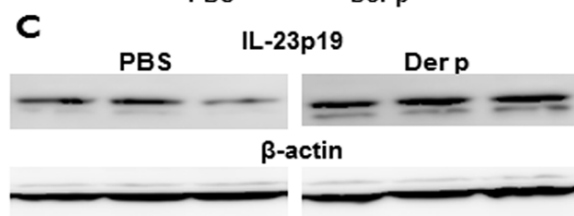
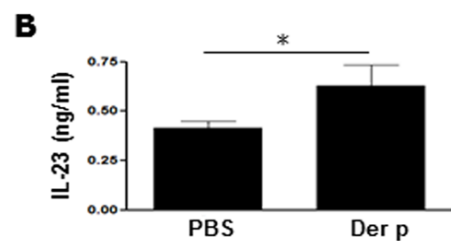
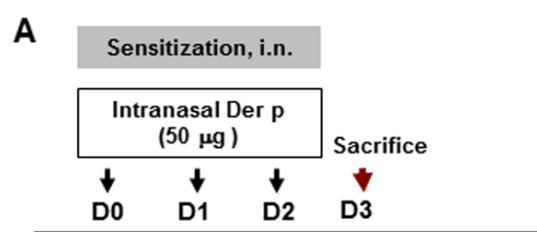
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Figure 6. Effects of anti IL-23 Ab on innate pro-Th2 cytokines and DC activation in Der p-stimulated MLE12 cells

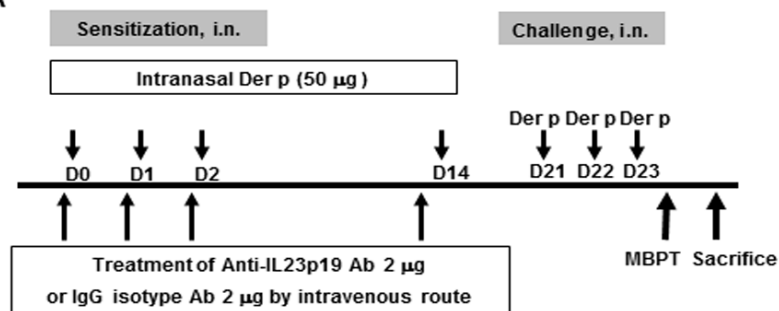
After, confluent MLE-12 cells were stimulated by Der p (80 μ g/ml), the protein level of IL-23

530 (A) and the mRNA expressions of IL-23p19 (B) and IL-23R (C) was determined by ELISA or real
531 time PCR. And MLE-12 cells were stimulated by Der p (80 µg/ml) and cultured for 24 h with or
532 without the presence of anti IL-23 Ab (0.05 µg/ml), anti IL-23R Ab (0.05 µg/ml), or recombinant IL-23
533 (0.01 µg/ml). The protein levels of IL-1α (D), GM-CSF (E) and expression of IL-33 (F) were
534 determined by ELISA or real time PCR. For co-cultivation, MLE-12 cells were treated each siRNA
535 (IL-23p19 siRNA, IL-23R siRNA, NC siRNA) or recombinant IL-23 for 6 h. And these MLE-12 cells
536 were stimulated by Der p (80 µg/ml) for 24 h. After wash with PBS, were co-cultured with BMDCs for
537 24 h. The numbers of CD11c+MHCII+ cells (G) and CD11c+CD86+ cells (H) in BMDCs were
538 determined using flow cytometry. * indicates $p < 0.05$, ** $p < 0.01$.

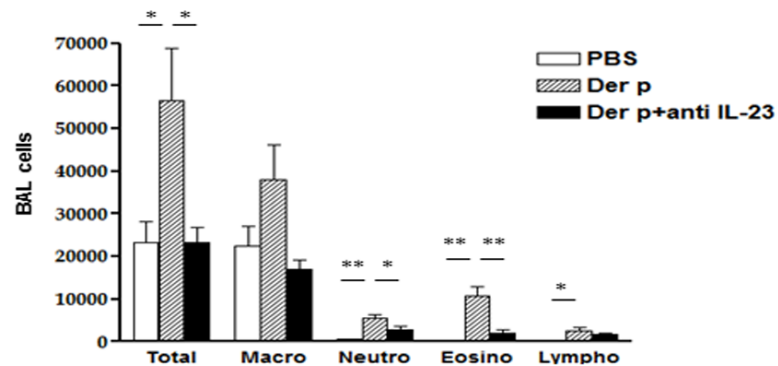
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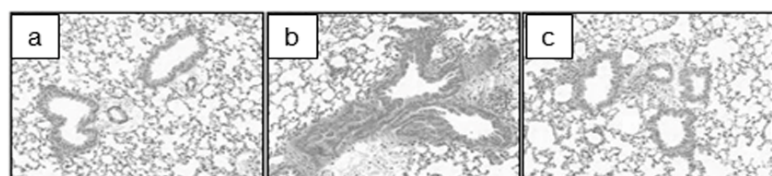
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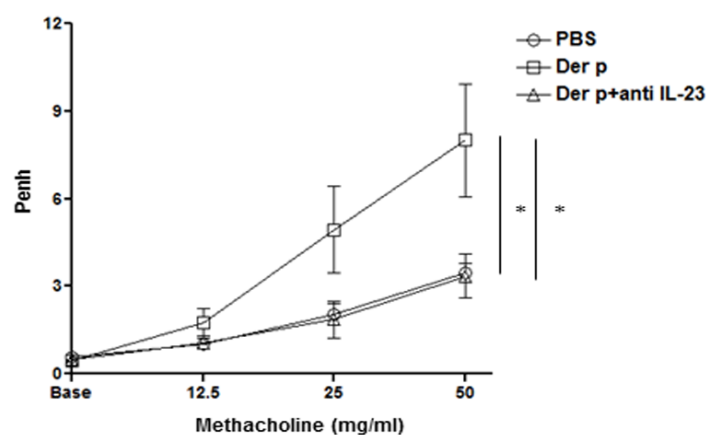
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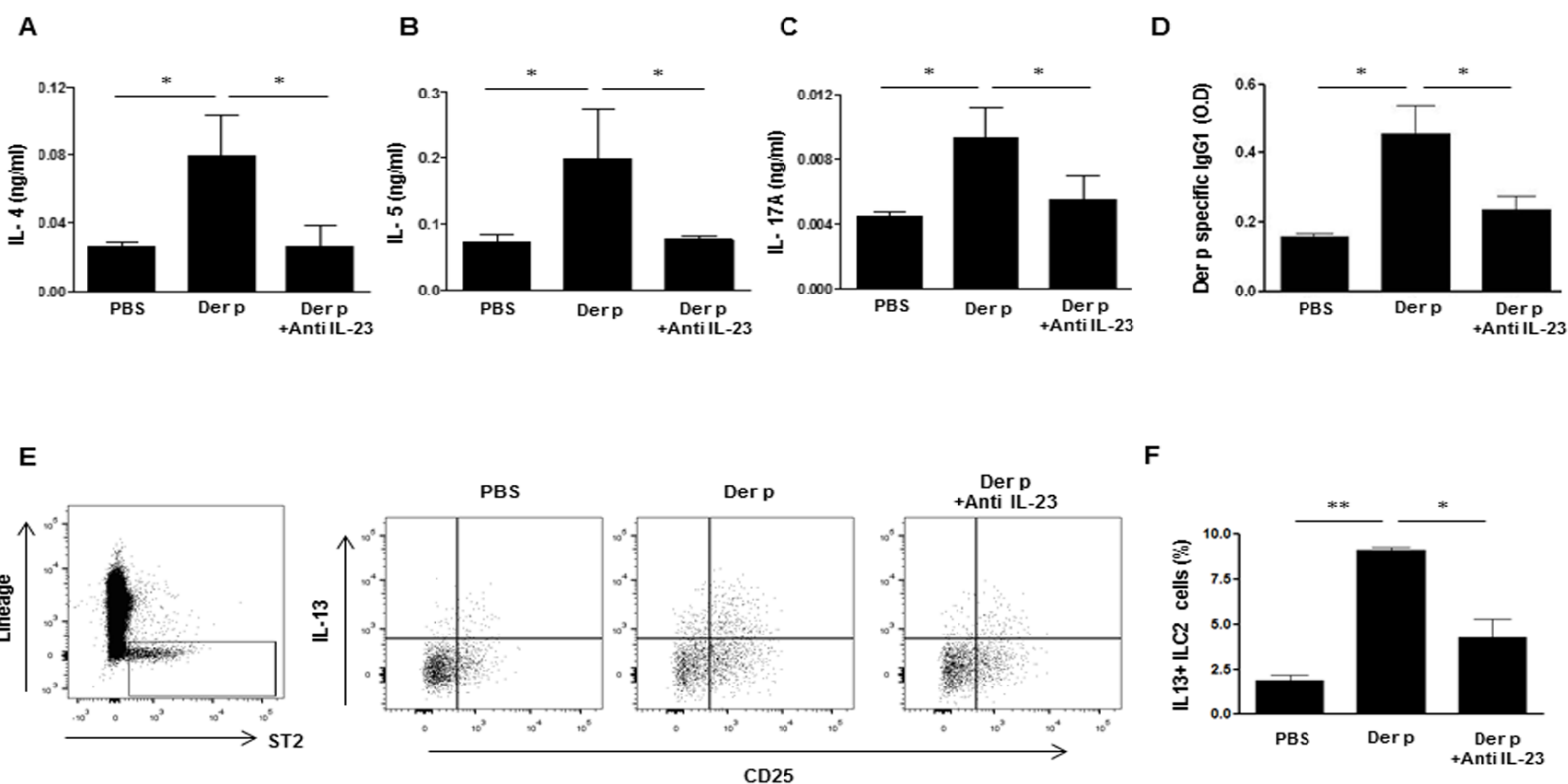


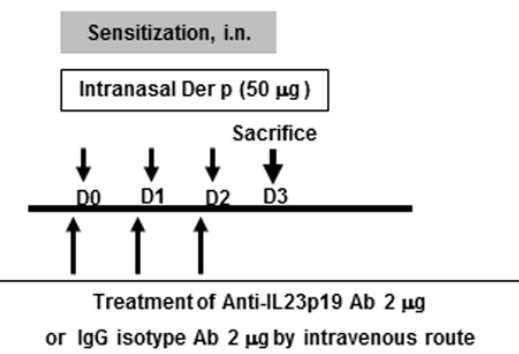
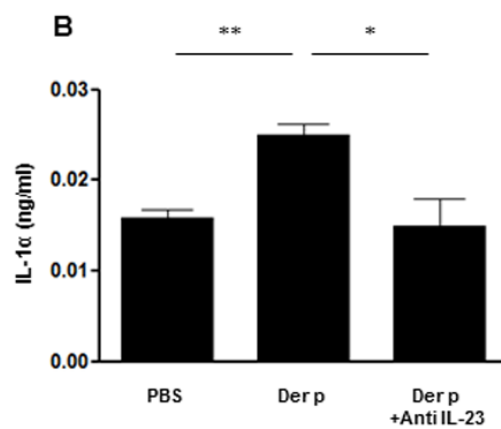
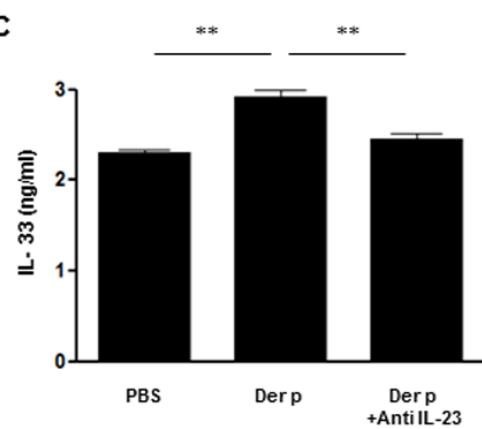
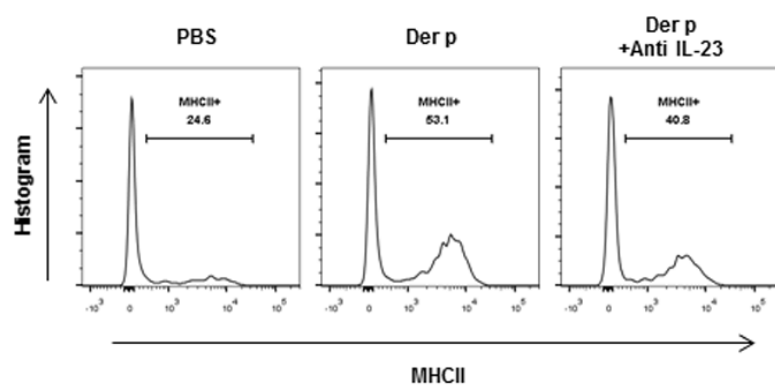
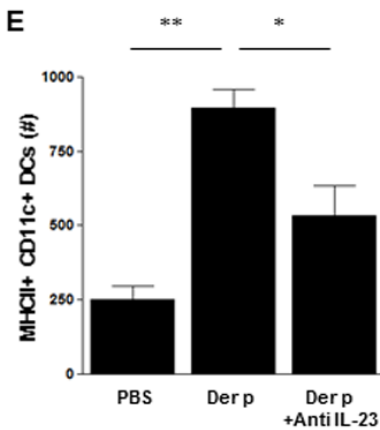
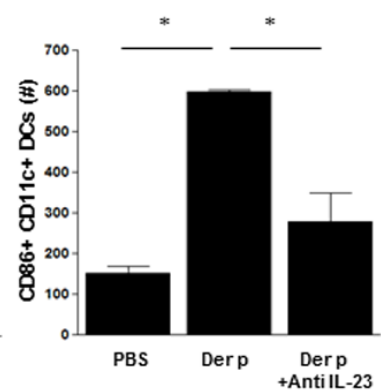
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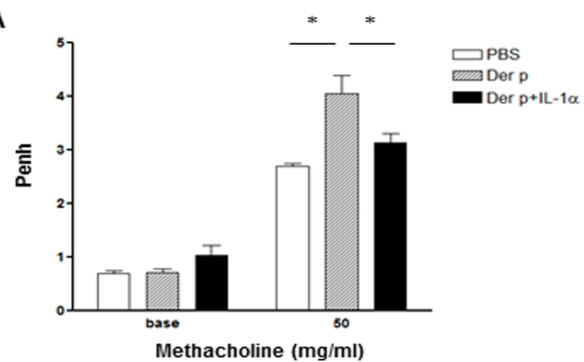
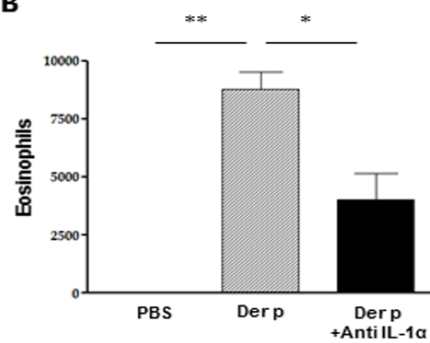
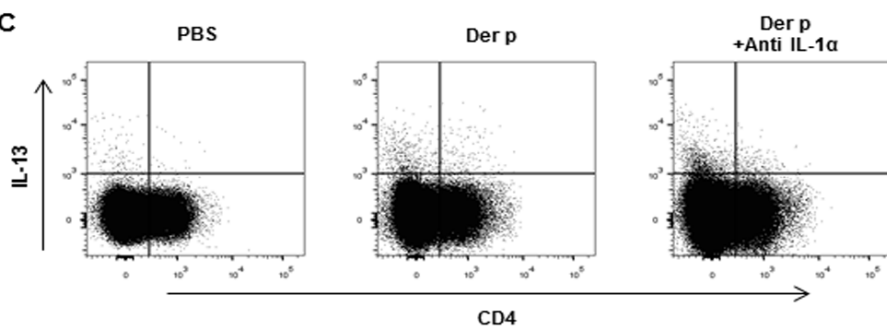
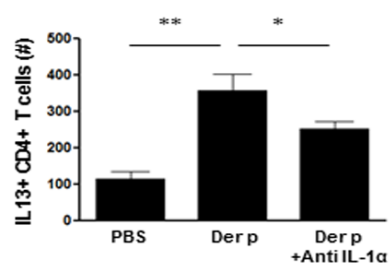
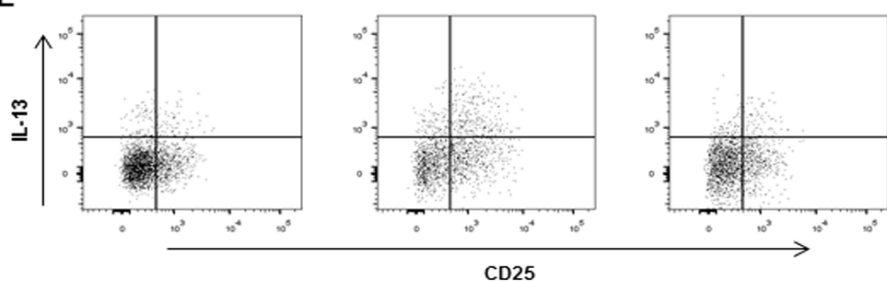


D





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