

Critical role of interleukin-5 in the development of a mite antigen-induced chronic bronchial asthma model

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Abstract

Objective and design Asthma is associated with eosinophilic airway inflammation and characterized by enhanced airway sensitivity. Interleukin (IL)-5 plays an important role in the pathogenesis of asthma. The involvement of IL-5 receptor-mediated cellular signals in the pathogenesis of a mite antigen-induced chronic asthma model was investigated.

Subjects In this study, 48 female C57BL/6J (WT) mice and IL-5 receptor-deficient (IL-5RKO) mice were used.

Treatment Mite antigen (50 µl) was intranasally administered 13 times to WT and IL-5RKO mice.

Methods Airway hypersensitivity (Mch PC₂₀₀) and specific antigen exposure tests were performed, and lung tissue, bronchoalveolar lavage fluid (BALF), and blood were collected to investigate the asthma pathology and differences in the local pulmonary levels of cytokines and chemokines.

Results Airway sensitivity was enhanced and antigen-specific airway resistance was increased in WT mice. In addition, the number of eosinophils and Th2 cytokine levels in the BALF were increased. In contrast, IL-5RKO mice did not acquire the asthma pathology, such as antigen-specific airway resistance and eosinophilic airway inflammation. Mch PC₂₀₀ was significantly correlated with cysteinyl leukotriene levels in WT mice.

Conclusion These findings suggested that both IL-5 induced eosinophils and cysteinyl leukotrienes are involved in the pathology of this mite antigen-induced chronic asthma model.

Keywords Asthma · *Dermatophagoides farinae* · IL-5 · IL-5 deficient mice · Cysteinyl leukotrienes

Introduction

Bronchial asthma is associated with eosinophilic airway inflammation and characterized by enhanced airway hyper sensitivity and smooth muscle contraction-induced airway obstruction [1, 2]. Various eosinophil-derived inflammatory mediators are involved in these pathologic conditions, such as major basic protein, eosinophil cationic protein, cysteinyl leukotrienes (CysLTs), radical oxygen species, and cytokines [3, 4]. Particularly, CysLTs have airway inflammation-inducing and airway-contracting actions [5], and their receptors are important targets for the treatment of bronchial asthma [6, 7]. Interleukin (IL)-5 is considered to play an important role in the production and maturation of eosinophils, and their migration to local sites [8].

Recent studies of the therapeutic effects of anti-IL-5 monoclonal antibody (mAb) treatment for bronchial asthma patients, however, revealed that while anti-IL-5 mAbs reduced the number of eosinophils in the blood and sputum, they had no effect on histamine-induced airway hypersensitivity or allergen provocation-induced late reactions [9], and the importance of eosinophilic inflammation for bronchial asthma was thus brought into question. More recently, inhibition of acute exacerbation, steroid dose reduction, and improvement of the quality of life by anti-IL-5 mAb treatment in patients with residual eosinophilic inflammation

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have been demonstrated [10, 11]. Now the relationship between IL-5-induced eosinophils and bronchial asthma has again begun to attract attention.

In the present study, we aimed to prepare a mouse chronic asthma model using a mite antigen, which is the most frequent antigen causing human bronchial asthma, rather than the conventionally used ovalbumin, as a disease model more closely reflecting the pathology of human bronchial asthma. In addition, we used Th1-dominant C57BL/6J mice rather than Th2-dominant BALB/c mice to prepare a model with a mode of development similar to that of human bronchial asthma, because of the Th1-dominant condition in human airways [12, 13]. Furthermore, C57BL/6J background IL-5 receptor-deficient (IL-5RKO) mice were similarly sensitized to investigate whether cellular reactions to IL-5 signals were directly involved in the development of bronchial asthma.

Materials and methods

Animal model

As a mouse bronchial asthma model, 8-week-old female C57BL/6J mice (WT) (Charles River Laboratory, Yokohama, Japan) sensitized by intranasal administration of mite antigen were designated as the sensitized group (WT/-Derf). Physiologic saline was administered to the control group (WT/-Saline). These groups were compared with regard to the following characteristics of bronchial asthma to confirm acquisition of the pathology: (1) eosinophilic inflammation of the airway, (2) enhancement of airway sensitivity, and (3) airway obstruction induced by exposure to specific antigen. In addition, 8-week-old female IL-5RKO mice [14] were similarly sensitized (IL-5RKO/-Derf, IL-5RKO/-Saline) at 8 weeks of age and compared to investigate the involvement of IL-5 in the pathogenesis. We prepared an asthma model in C57BL/6 mice by increasing the period and frequency of sensitization using a twofold increase in the volume of mites in an effort to overcome the obstacles in using mite antigen reported in a previous study [15]. Mite extract *Dermatophagoides farinae* (Derf) (LSL Co., Tokyo, Japan) was dissolved with distilled water and adjusted to 2 mg/ml with saline. Animals were anesthetized by intraperitoneal administration of 50 mg/kg pentobarbital sodium. After confirming adequate anesthesia, 50 μ l of 2-mg/ml Derf was administered into the nasal cavity. The antigen was administered 13 times at four times a week. Saline was similarly administered at the same volume and schedule to the control group. All experimental animals used in this study were under a protocol approved by the institutional animal care and use committee of Kawasaki Medical School.

Airway hypersensitivity test

Methacholine was adjusted to 6.25, 12.5, 25, 50, and 100 mg/ml and inhaled using a nebulizer. The methacholine concentration used was that at which the airway resistance increased two times (Mch PC₂₀₀) from baseline with saline inhalation. Airway resistance was measured for 2 min after a 3-min inhalation and subsequent 1-min rest (Pulmos; MIPS, Osaka, Japan). When Mch PC₂₀₀ was significantly lower than that in the saline-treated control group, the test result was judged as positive.

Specific antigen exposure test

Airway resistance was measured before and after nasal Derf administration, and the rate of increase of airway resistance from that before administration (Δ SRaw) was evaluated. When a significant elevation was observed compared to that in the saline-treated control group, the test was judged as positive.

Collection of blood, bronchoalveolar lavage, and lung tissue

Under respiratory arrest induced by intraperitoneal administration of pentobarbital sodium, blood and bronchoalveolar lavage (BAL) fluid were collected. BAL was performed with 1 ml of saline and the BAL fluid was centrifuged at 2,000g for 10 min, and cells were resuspended in 1 mL PBS and the total number of cells was determined using a hemocytometer. The cell suspension was cytocentrifuged (cytospin 3; Shandon, Astmoor, UK) on to microscope slides at 450 rpm for 6 min. The cytopins were stained using the Giemsa stain method. At least 300 cells, including eosinophils, neutrophils, lymphocytes, macrophages were counted differentially. Lung tissues were collected from some mice without BAL and were pathologically examined employing May–Giemsa staining and Kluver–Barrera's staining.

Enzyme-linked immunosorbent assay (ELISA)

IL-5, IL-13, IL-17, eotaxin, tumor necrosis factor- α , interferon- γ (R&D Systems, Minneapolis, MN, USA), and CysLTs (C4, D4, E4) (Cayman Chemical Company, Ann Arbor, MI, USA) in the bronchial alveolar lavage fluid (BALF) were measured using an ELISA. The detection limits were 15.6, 7.8, 10.9, 15.6, 10.9, 9.4 and 7.8 pg/ml for IL-5, IL-13, IL-17, eotaxin, tumor necrosis factor- α , interferon- γ , and CysLTs, respectively. The Derf-specific serum IgE and IgG1 were measured by ELISA as previously described [16].

Statistical analysis

Statistical analysis was performed using Stat View 5.0® (SAS Institute Inc., Cary, NC, USA). All data are expressed as mean $\pm$ standard error. Statistical analyses were performed by two-way analysis of variance, and Student's *t* test. The correlation between two elements was analyzed using Spearman's rank correlation coefficient. A *p* level of 0.05 was considered to indicate significance.

Results

Mch PC₂₀₀ was significantly lower in both WT/-Derf and IL-5RKO/-Derf compared to WT/-Saline and IL-5RKO/-Saline, respectively. Interestingly, airway hypersensitivity was significantly higher in WT/-Derf than in IL-5RKO/-Derf (Mch PC₂₀₀ 12.3 $\pm$ 1.77 vs. 28.5 $\pm$ 3.26 mg/ml, *p* < 0.01; Fig. 1a).

Regarding airway resistance after exposure to the specific antigen, the Δ SRaw was significantly higher in WT/-Derf than in WT/-Saline (25.8 $\pm$ 7.51 vs. 3.99 $\pm$ 0.54, *p* < 0.01). No significant difference was detected between IL-5RKO/-Derf and IL-5RKO/-Saline (4.39 $\pm$ 0.87 vs. 2.32 $\pm$ 0.55, *p* = 0.06). Δ SRaw was significantly greater in WT/-Derf than in IL-5RKO/-Derf (25.8 $\pm$ 7.51 vs. 4.39 $\pm$ 0.87, *p* < 0.01; Fig. 1b).

Numbers of inflammatory cells in the BALF were evaluated 24 h after the intranasal allergen challenge. After exposure to Derf, numbers of total leukocytes, lymphocytes, and neutrophils were significantly increased in BALF compared with saline-exposed WT and IL-5RKO mice, respectively (Fig. 2a), but there was no significant difference between WT/-Derf and IL-5RKO/-Derf (Fig. 2a). Interestingly, the eosinophil count differed significantly between WT/-Derf and IL-5RKO/-Derf (35.3 $\pm$ 31.7 vs. 0.08 $\pm$ 0.25 $\times 10^4$ /ml, *p* < 0.01; Fig. 2a). Similarly to the BAL findings, bronchial and lung perivascular eosinophil infiltration were observed in WT/-Derf, but no eosinophils were present in IL-5RKO/-Derf (Fig. 3a, b).

Comparisons of the cytokine and chemokine levels in the BALF are shown in Fig. 2b. The IL-5, IL-13, IL-17, and eotaxin levels were significantly elevated by Derf administration in WT, but no significant difference in cytokine levels was detected between WT/-Derf and IL-5RKO/-Derf, except in IL-5. The IL-5 level of WT/-Derf was significantly lower than that of IL-5RKO/-Derf (3.99 $\pm$ 0.26 vs. 25.9 $\pm$ 8.85 pg/ml, *p* < 0.01).

Derf administration significantly increased the leukotriene level in both WT and IL-5RKO. No significant difference was detected between WT/-Derf and IL-5RKO/-Derf, but the level tended to be higher in

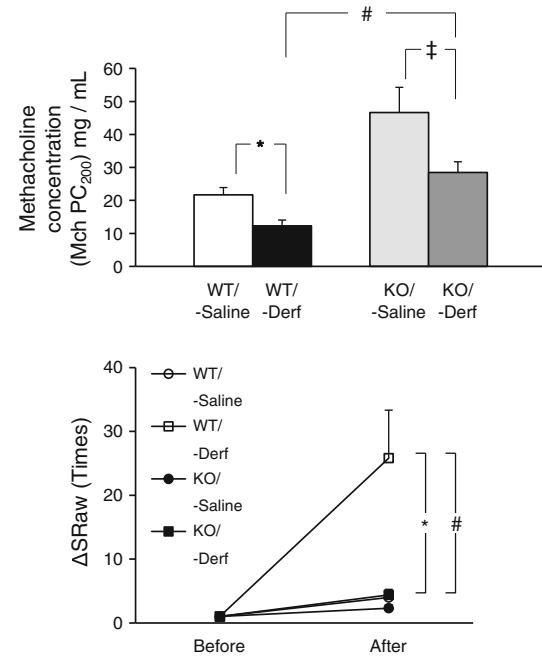


Fig. 1 Measurement of airway resistance in the mouse bronchial asthma model. **a** Airway hypersensitivity, airway resistance after methacholine inhalation was measured, and airway hypersensitivity is presented as Mch PC₂₀₀. The details are described in the “Materials and methods”. *Comparison between the WT/-Derf and WT/-Saline groups (*p* < 0.05). †Comparison between the IL5RKO/-Derf and IL5RKO/-Saline groups (*p* < 0.05). #: Comparison between the WT/-Derf and IL5RKO/-Derf groups (*p* < 0.05). **b** Elevation of antigen-specific airway resistance, airway resistance was measured before and after antigen challenge, and changes (Δ SRaw) were compared. *Comparison between the WT/-Derf and WT/-Saline groups (*p* < 0.05). #Comparison between the WT/-Derf and IL-5RKO/-Derf groups (*p* < 0.05). The measurement results are presented as the mean $\pm$ standard error in 5–10 mice. The significance of differences was analyzed using two-way ANOVA

WT/-Derf (6.11 $\pm$ 0.46 vs. 4.75 $\pm$ 0.54 ng/ml, *p* = 0.07; Fig. 2b).

Serum Derf-specific IgG₁ and IgE levels were significantly elevated by Derf, but no significant difference was detected between WT/-Derf and IL-5RKO/-Derf (Derf-specific IgG₁ 1.35 $\pm$ 0.12 vs. 1.10 $\pm$ 0.11 O.D., *p* = 0.10; Derf-specific IgE 0.23 $\pm$ 0.01 vs. 0.26 $\pm$ 0.02 O.D., *p* = 0.26, respectively; Fig. 4).

To investigate the factors involved in airway hypersensitivity, we analyzed the correlations between the parameters and Mch PC₂₀₀. The leukotriene level was significantly inversely correlated with Mch PC₂₀₀ in WT mice (*r* = −0.51, *p* < 0.05), but no significant correlation was detected in IL-5RKO mice (*r* = −0.43, *p* = 0.07). No correlation between Mch PC₂₀₀ and Derf-specific IgE was detected in WT or IL-5RKO mice (Fig. 5). No other parameter was significantly correlated with Mch PC₂₀₀ (data not shown).

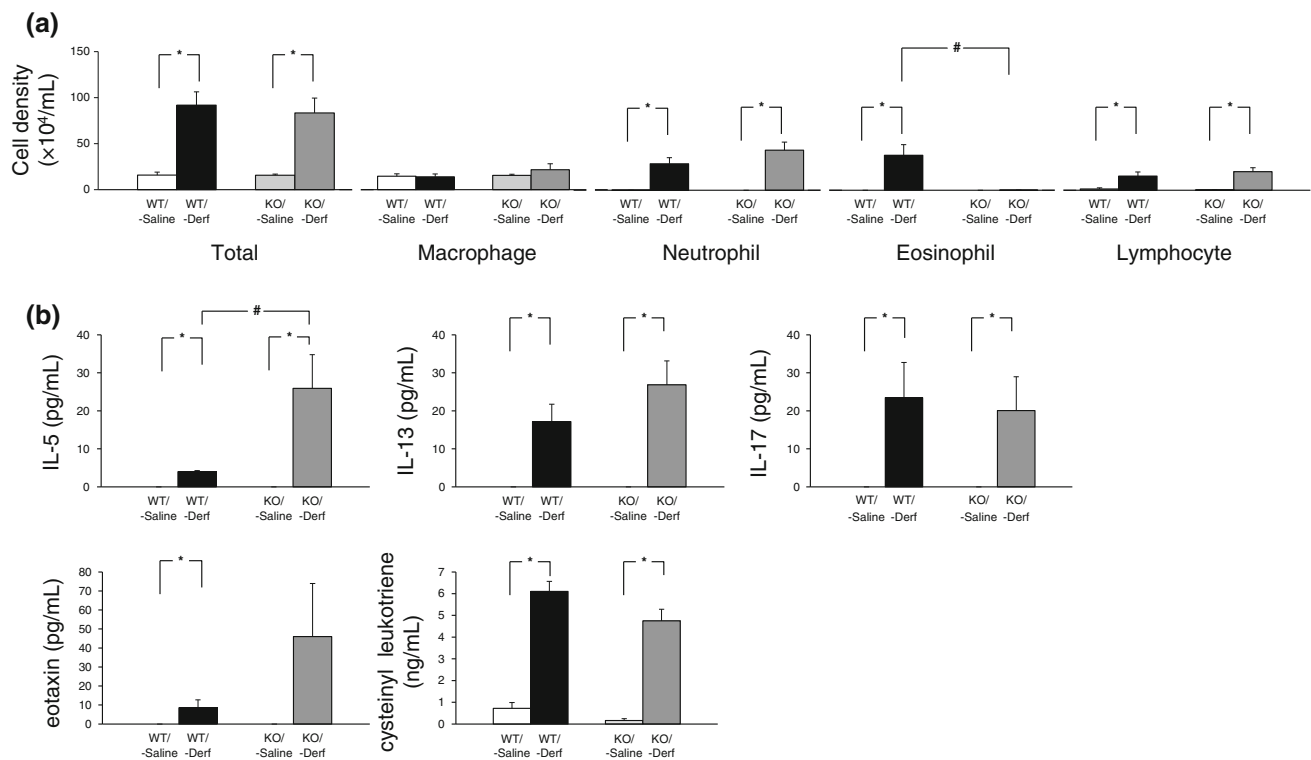


Fig. 2 Role of IL-5 in pulmonary eosinophilic inflammation. **a** Inflammatory cells in BALF were counted. The details are described in the “Materials and methods”. *Comparison between the Derf and Saline groups ($p < 0.05$). #Comparison between the WT/-Derf and IL-5RKO/-Derf groups ($p < 0.05$). **b** Comparison of cytokines and chemokines in BALF. The BALF IL-5, IL-13, IL-17,

eotaxin, and cysteinyl leukotriene levels were measured using an ELISA. *Comparison between the Derf and Saline groups ($p < 0.05$). The measurement results are presented as the mean $\pm$ standard error in 5–10 mice. The significance of differences was analyzed using two-way ANOVA

Discussion

Our mice model (WT/-Derf) intranasal Derf administrated without adjuvant had shown positive reactions to the specific antigen exposure and methacholine inhalation, and had increased eosinophil count in the BALF in WT/-Derf, confirming acquisition of the pathology of bronchial asthma. Furthermore, the Derf-specific IgE levels were slightly but significantly increased compared to that in WT/-Saline, and the Derf-specific IgG1 levels in WT/-Derf were extremely higher than that in WT/-Saline, indicating that this is a mouse Derf antigen-induced chronic bronchial asthma model resulting from 13 times antigen administrations. These findings suggest that the mode of the development of airway hypersensitivity in this mouse model is more similar to that of human bronchial asthma compared with the conventional ovalbumin-induced model and/or models using BALB/c [15].

Similar treatment also elevated the Derf-specific IgE and IgG1 levels in IL-5RKO: levels were significantly higher in IL-5RKO/-Derf than in IL-5RKO/-Saline, confirming sensitization with Derf. IL-5RKO/-Derf demonstrated a negative response to the specific antigen exposure test, and

the eosinophil count in the BALF was not significantly increased, indicating that the pathology of bronchial asthma was not acquired. These findings were consistent with previously reported findings observed in an ovalbumin-induced mouse BALB/c model [17], confirming the importance of IL-5 receptor-mediated cellular signals for the development of bronchial asthma in this Derf-induced C57BL/6J mouse model.

To search for the cause of airway hypersensitivity induced in this model, we examined the correlation with Mch PC₂₀₀. CysLTs in the BALF were moderately inversely correlated with Mch PC₂₀₀ in WT. In contrast, no significant correlation was detected in IL-5RKO. The main CysLT-producing cells are mast cells activated through IgE and eosinophils activated by IL-5 [18, 19]. Serum Derf-specific IgE levels did not differ significantly between WT/-Derf and IL-5RKO/-Derf ($p = 0.26$), whereas the eosinophil count in the BALF was significantly increased in WT/-Derf compared with IL-5RKO/-Derf ($p < 0.01$), and the CysLT levels in the BALF tended to be higher than that in IL-5RKO/-Derf ($p = 0.07$). These findings suggested that the difference in the correlation with CysLTs between WT and IL-5RKO might be due to involvement of

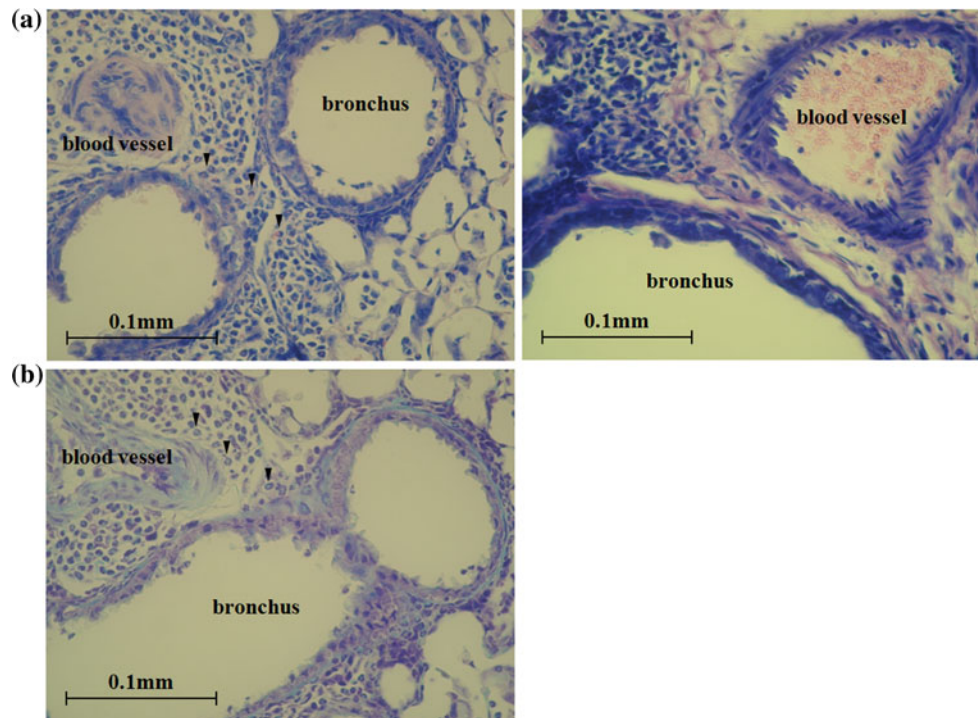


Fig. 3 Comparison of lung tissue. **a** May-Giemsa staining: *left* WT/-Derf group, *right* KO/-Derf group. Three animals were investigated in each group, and the typical histology is presented. Many eosinophils (*filled arrowhead*) are infiltrated into the circumference of a bronchus in WT/-Derf group, but inflammatory cells (not eosinophils)

in KO/-Derf group. Only three *filled arrowheads* are represented. The cytoplasm of the eosinophil has stained red. **b** In Kluver-Barrera's staining, the cytoplasm of an eosinophil had stained blue. The cells expected to be eosinophils by May-Giemsa staining were confirmed as eosinophils by Kluver-Barrera's staining

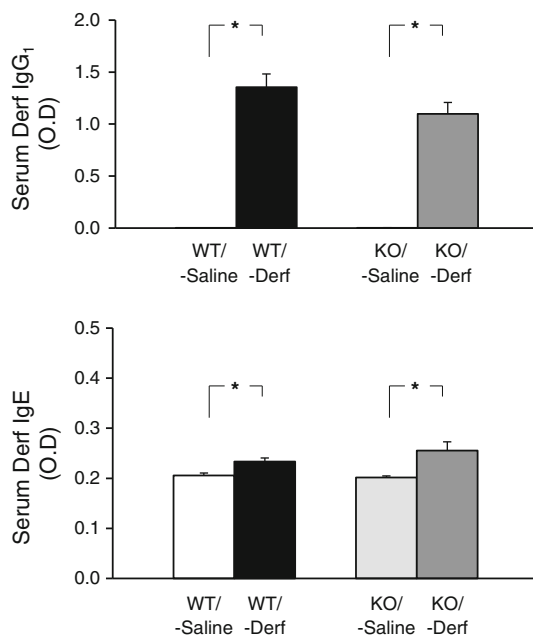


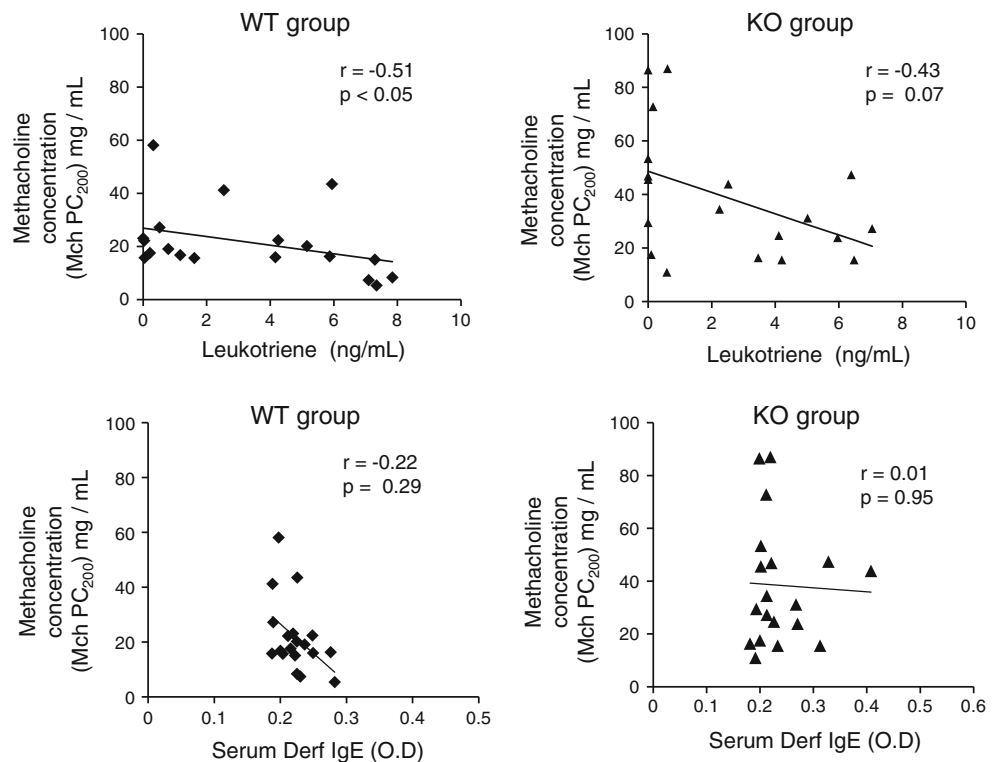
Fig. 4 Comparison of serum mite antigen-specific IgE and IgG₁. The serum Derf specific-IgE and -IgG₁ levels were measured. The details are described in the “[Materials and methods](#)”. *Comparison between the Derf and Saline groups ($p < 0.05$). The measurement results are presented as the mean $\pm$ standard error in 5–10 mice. The significance of differences was analyzed using two-way ANOVA

eosinophil-derived CysLTs, not mast cell-derived CysLTs. Further investigations are required to elucidate the mechanisms of airway hypersensitivity. Interestingly, there were some inconsistencies between the results of the airway hypersensitivity test and the specific antigen exposure test in IL-5RKO/-Derf. Eosinophil production, maturation, and migration to local sites induced by IL-5 receptor-mediated cellular signals might be partially involved in the development of airway hypersensitivity. Airway resistance 20 min after exposure to the specific antigen was not significantly elevated in IL-5RKO/-Derf compared with that in IL-5RKO/-Saline. Not only IgE-stimulated mast cells, but also IL-5 activated eosinophils might play an important role in early-phase asthmatic responses.

In the BALF of the IL-5RKO/-Derf group, there were few eosinophils, whereas eotaxin levels were elevated. Eotaxin accumulates eosinophils into the airway in combination with IL-5. In the IL-5RKO/-Derf group, eotaxin could not accumulate eosinophils into the airway without an IL-5 signal.

Fattouh et al. [20] reported that a defect involving eosinophils induced airway hypersensitivity in a BALB/c mouse Df-induced model similarly to that in wild-type mice. Airway hypersensitivity is also induced in eosinophil-deficient BALB/c mice, but not in eosinophil-deficient

Fig. 5 Correlation with Mch PC₂₀₀. The correlations between Mch PC₂₀₀ and the BALF cysteinyl leukotriene level (*top*) and between Mch PC₂₀₀ and the serum Derf-specific IgE titer (*bottom*) were analyzed in the WT and IL-5RKO groups using Spearman's rank correlation coefficient. The significance level was set at $p < 0.05$



C57BL/6 mice, showing the necessity of eosinophils for the induction of airway hypersensitivity, although the antigen was ovalbumin in these models [21]. These findings suggest that the importance of eosinophils to acquire the pathology of bronchial asthma is determined by the mouse strain and the knockout target. To elucidate the pathology of bronchial asthma and develop a therapeutic drug, it is essential to create a mouse model in which bronchial asthma develops through a mode close to the natural course of human bronchial asthma.

In conclusion, we succeeded in establishing a mouse model of bronchial asthma using a Derf antigen and C57BL/6J mice that develops via a mode closer to that of the natural course of human bronchial asthma than other current models. The importance of IL-5 receptor-mediated cellular signals to acquire the pathology of bronchial asthma was confirmed in this model, and the involvement of eosinophils and CysLTs in airway hypersensitivity was suggested as a possible mechanism. Further studies are required to clarify the association of eosinophils with CysLTs. The findings of this model may be useful for analyzing the pathology of bronchial asthma and developing therapeutic drugs.

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Conflict of interest The authors declare no financial or commercial conflict of interest.

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