

ORIGINAL ARTICLE

Pathogenesis of allergen-induced eosinophilic esophagitis is independent of interleukin (IL)-13

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Several studies have shown that interleukin (IL)-13 is induced in the esophageal biopsies of eosinophilic esophagitis (EoE) patients and promotes esophageal eosinophilia in mice, following an IL-13 challenge. However, the role of IL-13 has not been clearly investigated in allergen-induced EoE. Accordingly, we tested the hypothesis that IL-13 is required in allergen-induced EoE. Mice deficient in IL-13, STAT (signal transducer and activator of transcription)6 and both IL-4/IL-13 genes with their respective controls were challenged with *Aspergillus* extract, and IL-5 gene deficient with their control were challenged with recombinant IL-13, intranasally. The lung and esophageal eosinophils, mast cells and collagen accumulation were examined. Herein, we report that intranasal delivery of IL-13 promotes IL-5-dependent esophageal eosinophilia. However, allergen-induced EoE is not impaired in the IL-13 gene-deficient mice. In addition, wild-type and IL-13 gene-deficient mice demonstrated a comparable level of mast cells and collagen accumulation in the esophagus, following allergen-induced experimental EoE. Similarly, we found that esophageal eosinophilia in IL-4/IL-13 double gene-deficient and STAT6 gene-deficient mice were also not reduced following allergen-induced experimental EoE. In contrast, lung eosinophilia was significantly reduced in mice deficient in IL-13, both IL-4/IL-13 and STAT6 genes following allergen challenge. In conclusion, our data establish that allergen-induced EoE pathogenesis is independent of IL-13, whereas IL-13 is required for allergen-induced lung eosinophilia. *Immunology and Cell Biology* (2013) 91, 408–415; doi:10.1038/icb.2013.21; published online 21 May 2013

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Eosinophilic esophagitis (EoE) is a painful and sometimes devastating inflammatory disease of the esophagus that often leads to swallowing problems, food refusal, food intolerance in infants, dysphagia and food impaction in adolescents and adults.^{1–4} Both pediatric and adult EoE patients develop fibrosis and other anatomical complications including esophageal strictures.^{4–12} EoE is now considered a global health problem for children in multiple developed and developing countries over the last decade.^{1,5,12–19} EoE is associated with allergic responses; for example, patients with EoE have a high rate of atopy and their clinical symptoms, and eosinophilic infiltrations are ameliorated by an elemental diet or by anti-inflammatory glucocorticoid therapy.^{20,21} Interestingly, interleukin (IL)-13 appears to be particularly important, as it is produced in high quantities by Th2 cells and regulates multiple characteristics of allergic diseases.²² The levels of elevated IL-13 is an important regulator of a number of allergic diseases including asthma,²³ EoE,^{24–28} atopic dermatitis^{29–31} and allergic rhinitis.^{32,33} IL-13 share a common receptor subunit with the IL-4 R α , and signals through the STAT (signal transducer and activator of transcription)6.³⁴ Th2 cells produce the cytokine IL-5, which is specific for the growth and survival of eosinophils. We demonstrated earlier that IL-5 is overexpressed in the esophagus of

patients with EoE³⁵ and systemic overexpression of IL-5 (via pharmacological or transgenic approaches) promotes EoE in mice.³⁶ It has been previously shown that *in vitro* IL-13 activates esophageal epithelial cells and induces eosinophil chemokines, eotaxin-1, -2 and -3.³⁷ Additionally, it has also been shown that IL-13 induces IL-5 that may be responsible for IL-13-induced tissue eosinophilia.²⁶ Therefore, it is important to understand the role of IL-13 in promoting esophageal eosinophilia whether IL-13 directly acts and promotes esophageal eosinophilia, or esophageal eosinophilia is due to the induction of IL-5 and eotaxins. Of note, mice with targeted deletion of IL-13, both IL-4/IL-13, or STAT6 develop attenuation of certain features of allergic disease, like asthma.^{38,39} Further, we also previously showed that allergen challenge promotes IL-5-mediated experimental asthma and EoE in mice.⁴⁰ The allergen-induced experimental EoE in mice mimic most of the characteristic features observed in individuals with various forms of EoE, such as intra-epithelial eosinophils, extracellular granule deposition and epithelial cell hyperplasia.⁴⁰ Importantly, overexpression of IL-13 by transgenic approaches induces multiple features of EoE, including eosinophilia, collagen deposition and reduced lumen circumference.^{25,41} Therefore, it is

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rationale to know whether IL-13 is directly responsible for allergen-induced EoE pathogenesis. Accordingly, we tested the hypothesis that IL-13 is critical in the induction and progression of EoE. Therefore, we delivered *Aspergillus* allergen to the IL-13, both IL-4/IL-13 and their signaling molecule STAT6 gene-deficient mice. The data presented in this manuscript establish that IL-13 intranasal delivery promote IL-5-dependent esophageal eosinophilia; however, IL-13 signaling is not critical in promoting intranasal allergen-associated EoE pathogenesis.

RESULTS

Intranasal IL-13 induces IL-5-mediated esophageal eosinophilia

We were first interested in determining if intranasal delivery of IL-13 induces EoE. In order to test this, 10 g of recombinant IL-13 was delivered intranasally to the wild-type mice three times on alternate days. Mice received three doses of recombinant IL-13 10 µg per 40 µl (or 40 µl saline) at an interval of 24 h, and the eosinophil level in the esophagus was determined 24 h after the last IL-13 delivery. IL-13 administration to Balb/c mice promotes esophageal eosinophilia. As a control, mice treated with intranasal saline did not have significant levels of esophageal eosinophils. The eosinophil levels in the esophagus of IL-13 and saline-treated mice were 24.3 ± 4.6 and $1.1 \pm 1.6 \text{ mm}^{-2}$, following three intranasal IL-13 treatments (mean $\pm$ s.d., $n = 6$; $P < 0.001$), respectively. Next, we tested whether IL-13-induced esophageal eosinophilia is due to IL-5 induction because it has been shown that IL-13 induces IL-5.²⁶ We addressed this by treating IL-5 gene-targeted mice by intranasal IL-13. IL-13 intranasal treatment to IL-5 gene-deficient mice did not develop esophageal eosinophilia; whereas wild-type control mice developed marked esophageal eosinophilia (Figure 1a). The eosinophil levels in the esophagus of wild-type and IL-5 gene-deficient mice, following three doses of 10 g IL-13 treatment on alternate days were 23.1 ± 8.4 and $1.3 \pm 1.7 \text{ mm}^{-2}$ (mean $\pm$ s.d., $n = 4-5$; $P < 0.001$), whereas saline-treated wild-type and IL-5 gene-deficient mice had 1.1 ± 1.4 and $0.9 \pm 1.2 \text{ mm}^{-2}$, respectively. For comparison, eosinophil levels in the bronchoalveolar lavage fluid (BALF) of wild-type and IL-5 gene-targeted mice following three doses of 10 µg intranasal IL-13 were $10.6 \pm 2.7 \times 10^4$ and $0.57 \pm 0.48 \times 10^4$ per lung (mean $\pm$ s.d., $n = 6-8$; $P < 0.001$), whereas saline-treated wild-type and IL-5 gene-deficient mice had $0.005 \pm 0.01 \times 10^4$ per lung and $0.002 \pm 0.004 \times 10^4$ per lung, respectively (Figure 1b). Collectively, these results establish an essential role for IL-5 in IL-13-induced esophageal eosinophilia. These data are similar to our earlier reported intratracheal-delivered IL-13 findings;²⁵ but to compare *Aspergillus*-induced EoE to IL-13-delivered EoE, it was needed to repeat the experiment by delivering IL-13 with a similar route.

IL-13 gene-deficient mice do not induce allergen-induced EoE

Next, we tested the hypothesis that allergen-induced EoE is IL-13-dependent. Therefore, we induced experimental allergen-induced EoE in IL-13 gene-deficient mice, and wild-type control mice were challenged with *Aspergillus* antigen. As a negative control, both types of mice were challenged with saline. The eosinophil levels in the esophagus of *Aspergillus*-challenged wild-type and IL-13-deficient mice were 43.8 ± 9.1 and $41.8 \pm 10.5 \text{ mm}^{-2}$ (mean $\pm$ s.d., $n = 8$; $P < 0.1$), whereas in saline-treated mice they were 1.3 ± 1.8 and $1.2 \pm 1.7 \text{ mm}^{-2}$ (mean $\pm$ s.d., $n = 8$), respectively. As a comparison, eosinophil levels in the BALF were also examined. The eosinophils in the BALF of *Aspergillus*-challenged wild-type mice were $23.2 \pm 3.2 \times 10^4$ per lung (mean $\pm$ s.d., $n = 8$) compared with $6.6 \pm 2.8 \times 10^4$ per lung in IL-13 gene-deficient mice (mean $\pm$ s.d.,

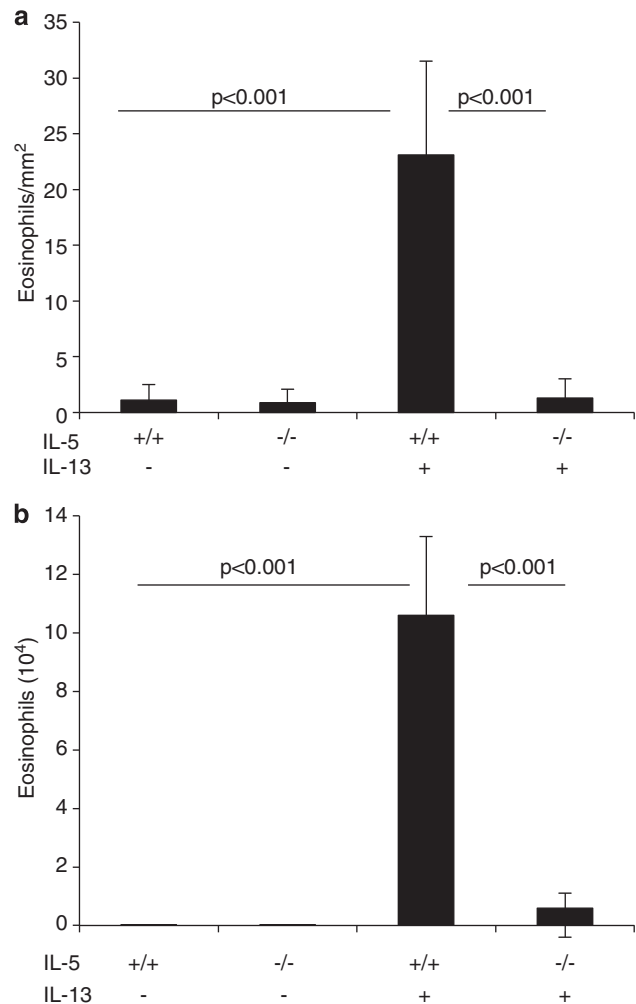


Figure 1 IL-13-induced esophageal eosinophilia in wild-type and IL-5 gene-targeted mice. The level of eosinophils in the esophagus and lung of wild-type and IL-5 gene-targeted mice were analyzed following intranasal 10 µg of IL-13 challenge. Wild-type (+/+) or IL-5-deficient (-/-) mice were challenged and eosinophils were determined by anti-MBP staining in the esophagus (a), and in the lung fluid (b) by differential counting of cells in BALF. The results are expressed as mean $\pm$ s.d. ($n = 8$ mice per group).

$n = 8$; $P < 0.01$); whereas saline-treated wild-type and IL-13 gene-deficient mice had $0.01 \pm 0.01 \times 10^4$ and $0.01 \pm 0.01 \times 10^4$ per lung (mean $\pm$ s.d., $n = 8$), respectively (Figures 2a and b). Furthermore, most of the EoE characteristics such as the induction of intra-epithelial eosinophilia (Figures 3a and b), esophageal mast cells (Figures 3c and d) and lamina propria collagen (Figures 3e and f) were found in comparable levels in both wild-type and IL-13 gene-deficient mice, following induction of allergen-induced experimental EoE. The quantitation of collagen thickness and mast cell numbers were performed and found comparable in allergen-challenged wild-type and IL-13 gene-deficient mice (Figures 3g and h). Collectively, these results establish that IL-13 is not critical in allergen-induced EoE. We also tested the role of IL-4 in allergen-induced EoE by using IL-4Rα gene-deficient mice, and similar data to IL-13 gene-deficient mice were found (data not shown). This is not surprising as both IL-4 and IL-13 are related cytokines that share a common signal transduction mechanism involving the IL-4 receptor alpha chain and STAT6.

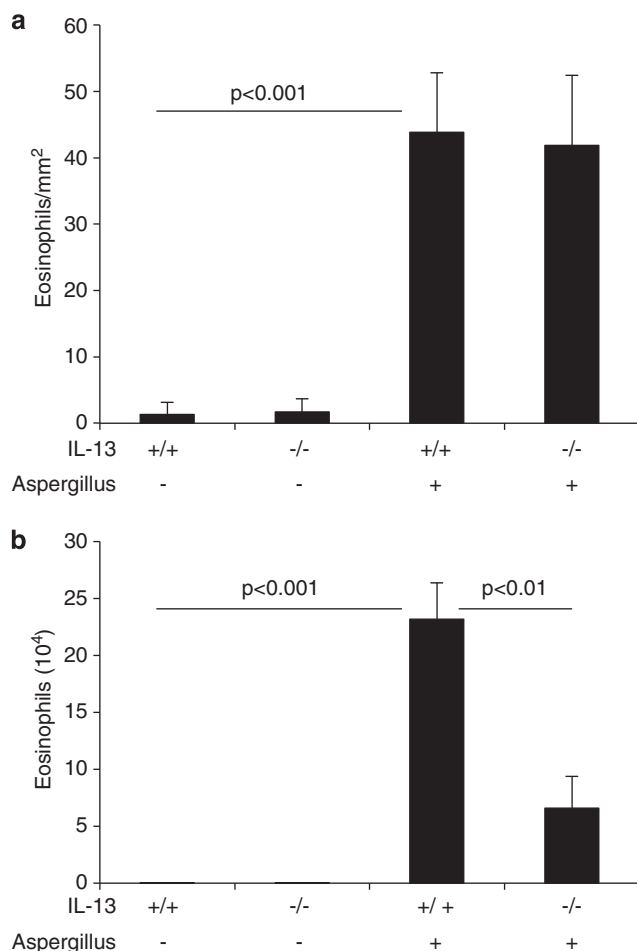


Figure 2 *Aspergillus*-induced eosinophilia in IL-13 gene-targeted mice. The level of eosinophils in the esophagus and lung of wild-type and IL-13 gene-targeted mice were analyzed following intranasal nine doses of *Aspergillus* antigen challenge in 3 weeks of allergen challenge regime. Wild-type (+/+) or IL-13-deficient (-/-) mice were challenged and eosinophils were determined by anti-MBP staining in the esophagus (a), and in the lung fluid (b) by differential counting of cells in BALF. The results are expressed as mean $\pm$ s.d. ($n = 12$ mice per group).

IL-4/IL-13 double gene-deficient mice are not protected from the induction of allergen-induced EoE

Next, we tested the hypothesis that allergen-induced EoE is IL-4/IL-13-dependent. IL-4/IL-13 has been shown to have a significant role in allergen-induced mouse model of experimental asthma.^{42,43} Accordingly, IL-4/IL-13 double gene-deficient mice and strain-matched wild-type control mice were challenged with saline or *Aspergillus* antigen. The eosinophil levels in the esophagus of *Aspergillus*-challenged wild-type and IL-4/IL-13 double gene-deficient mice were 46.5 ± 16.4 and $28.9 \pm 7.3 \text{ mm}^{-2}$ (mean $\pm$ s.d., $n = 8$; $P < 0.08$), whereas in saline-treated mice they were 0.6 ± 1.3 and $1.1 \pm 1.5 \text{ mm}^{-2}$ (mean $\pm$ s.d., $n = 8$), respectively. As a comparison, eosinophil levels in the BALF were also examined. The eosinophils in the BALF of *Aspergillus*-challenged wild-type mice were $63.9 \pm 51.5 \times 10^4$ per lung (mean $\pm$ s.d., $n = 8$) compared with $12.8 \pm 0.5 \times 10^4$ per lung in IL-4/IL-13 double gene-deficient mice (mean $\pm$ s.d., $n = 8$; $P < 0.001$); whereas saline-treated wild-type and IL-4/IL-13 double gene-deficient mice had $0.01 \pm 0.1 \times 10^4$ and $0.01 \pm 0.1 \times 10^4$ per lung (mean $\pm$ s.d., $n = 8$), respectively

(Figures 4a and b). Collectively, these results further establish that allergen-induced EoE is not dependent on IL-4/IL-13.

Aspergillus-induced EoE is STAT6-independent

We were next interested in determining the role of STAT6 in regulating EoE. STAT6 has been shown to be important in regulating allergen-induced IL-4- and IL-13-mediated eosinophils in the lung, but its effect in the gastrointestinal tract has only been studied in the intestine following parasitic infection.⁴⁴ Accordingly, we tested the role of STAT6 in the development of allergen-induced EoE. STAT6 gene-deficient mice and strain-matched wild-type control mice were challenged with saline or *Aspergillus* antigen. The eosinophil levels in the esophagus of *Aspergillus*-challenged wild-type and STAT6-deficient mice were 51.4 ± 9.1 and $34.1 \pm 8.8 \text{ mm}^{-2}$ (mean $\pm$ s.d., $n = 8$; $P < 0.07$), whereas in saline-treated mice they were 1.4 ± 1.7 and $1.7 \pm 2.1 \text{ mm}^{-2}$ (mean $\pm$ s.d., $n = 8$), respectively. As a comparison, eosinophil levels in the BALF were also examined. The eosinophils in the BALF of *Aspergillus*-challenged wild-type mice were $14.4 \pm 3.7 \times 10^4$ per lung (mean $\pm$ s.d., $n = 8$) compared with $5.6 \pm 1.6 \times 10^4$ per lung in STAT6 gene-deficient mice (mean $\pm$ s.d., $n = 8$; $P < 0.01$); whereas saline-treated wild-type and STAT6 gene-deficient mice had $0.01 \pm 0.01 \times 10^4$ and $0.01 \pm 0.01 \times 10^4$ per lung (mean $\pm$ s.d., $n = 8$), respectively (Figures 5a and b). Collectively, these results establish that *Aspergillus*-induced EoE is not dependent on STAT6.

Analysis of eosinophil-active cytokine, IL-5 and chemokines eotaxin-1 and eotaxin-2 transcript levels in wild-type, IL-4/IL-13 double gene-deficient and STAT6 gene-deficient mice

Next, we examined *eotaxin-1*, *eotaxin-2*, *IL-5* and *GADPH* mRNA expression in the lung and esophagus of saline- and *Aspergillus*-challenged wild-type, IL-4/IL-13 double gene-deficient and STAT6 gene-deficient mice. Our quantitative polymerase chain reaction (PCR) analysis showed that *Aspergillus*-challenged mice had a significant increase of the relative mRNA expression of esophageal and lung *eotaxin-1*, *eotaxin-2* and *IL-5* compared with the saline-challenged wild-type mice (Figures 6a–f). However, a comparable transcript expression level of *eotaxin-1* and *IL-5* were found in allergen-challenged wild-type and IL-4/IL-13 double gene-deficient mice (Figures 6a and c), and allergen-challenged wild-type and STAT6 gene-deficient mice (Figures 6d and f). The transcript expression level of *eotaxin-2* was significantly reduced in the lungs, but not in the esophagus of allergen-challenged IL-4/IL-13 double gene-deficient and STAT6 gene-deficient mice compared with their respective wild-type mice (Figures 2b and e). These data indicate that reduced *eotaxin-2* transcript in the lung may be responsible for significantly reduced lung eosinophilia in IL-4/IL-13 double gene-deficient and STAT6 gene-deficient mice.

DISCUSSION

Eosinophil accumulation in the esophagus is characteristic of a variety of clinical disorders including GERD, eosinophilic gastroenteritis and EoE.^{2,45–49} Recent clinical studies have suggested that the prevalence of these disorders, especially EoE, is increasing frequency.^{48,50} It has been reported that IL-13 overexpression is associated with the induction of esophageal eosinophilia and fibrosis, which is commonly observed in the human and experimental model of EoE.⁴⁰ Herein, we first confirm our previous findings that intranasal IL-13 also promote a similar levels of eosinophil accumulation in the esophagus, like intratracheally delivered IL-13.^{25,41} However, the significance of IL-13 in allergen-induced

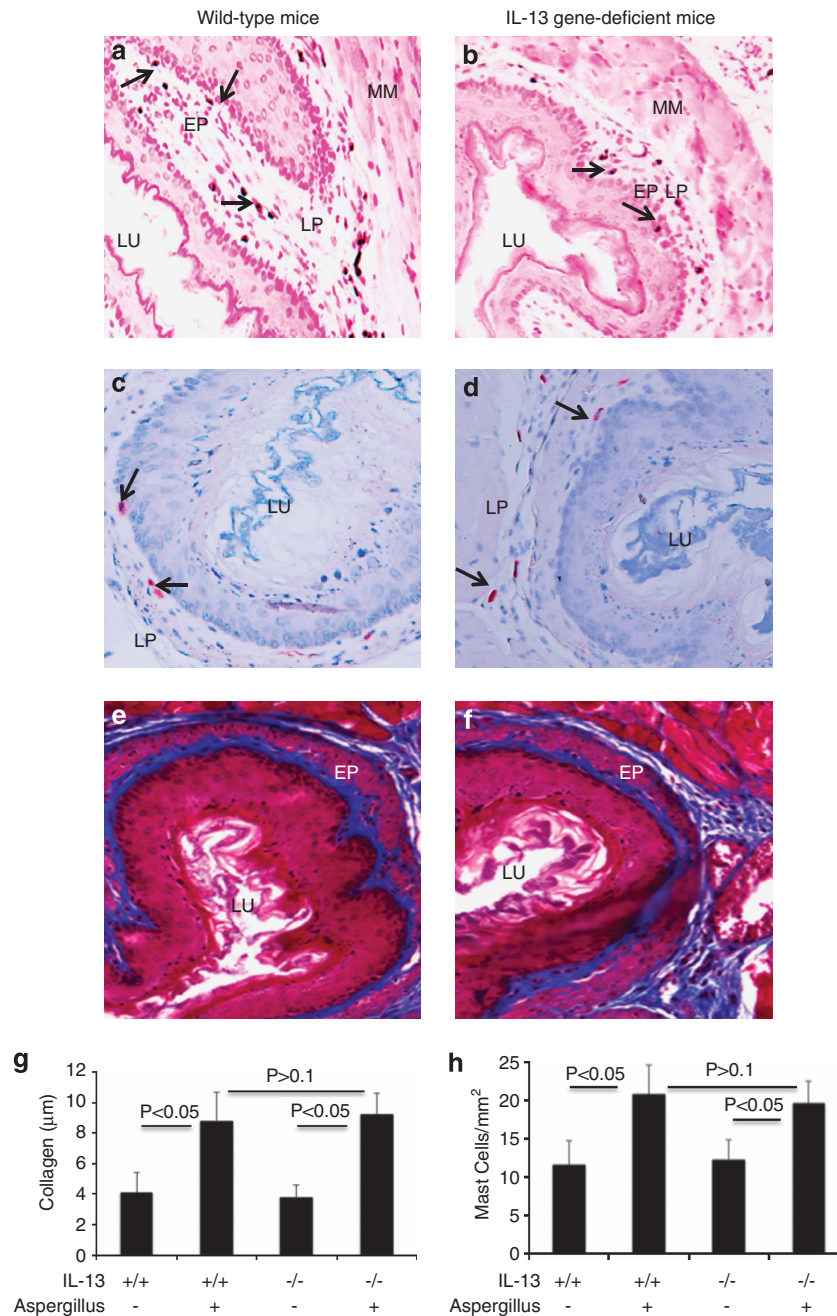


Figure 3 Detection of intra-epithelial eosinophils, mast cells and collagen in IL-13 gene-deficient mice following *Aspergillus*-induced EoE. The EoE characteristic features were examined in wild-type and IL-13 gene-deficient mice following the induction of *Aspergillus* antigen-induced experimental EoE. Both wild-type and IL-13 gene-deficient mice show intra-epithelial eosinophils (a and b, original magnification $\times 200$), mast cells (c and d, original magnification $\times 200$), and lamina propria and epithelial mucos collagen accumulation (e and f, original magnification $\times 400$). Arrows indicate intra-epithelial eosinophils or lamina propria mast cells in the respective photomicrographs. The quantitation of collagen thickness and mast cell numbers in wild-type and IL-13 gen-deficient mice are shown (g and h). The results are expressed as mean $\pm$ s.d. ($n = 8$ mice per group). EP-epithelium, LP-lamina propria, LU-lumen, MM-muscular mucosa.

EoE was not earlier proven. Notably, EoE is an allergen-induced esophageal disorder.^{20,21} Therefore, the present study was designed to test the hypothesis that IL-13 is critical in allergen-induced EoE pathogenesis. We show for the first time that intranasal IL-13 induces IL-5-dependent esophageal eosinophilia. These findings are in accordance with the earlier report that indicate IL-5 is required in IL-13-induced eosinophilia in the lung.²⁶ In this report, we first time show that allergen-induced EoE is independent to IL-13 in

experimental model because IL-13 gene-deficient mice are not protected from the development of allergen-induced EoE. Both wild-type and IL-13 gene-deficient mice show comparable levels of esophageal eosinophils and induced mast cells. Additionally, we show that both wild-type and IL-13 gene-deficient mice have most of the EoE characteristic features, like comparable intra-epithelial eosinophils, induced and lamina propria collagen accumulation following allergen challenge. In contrast, the allergen-induced lung

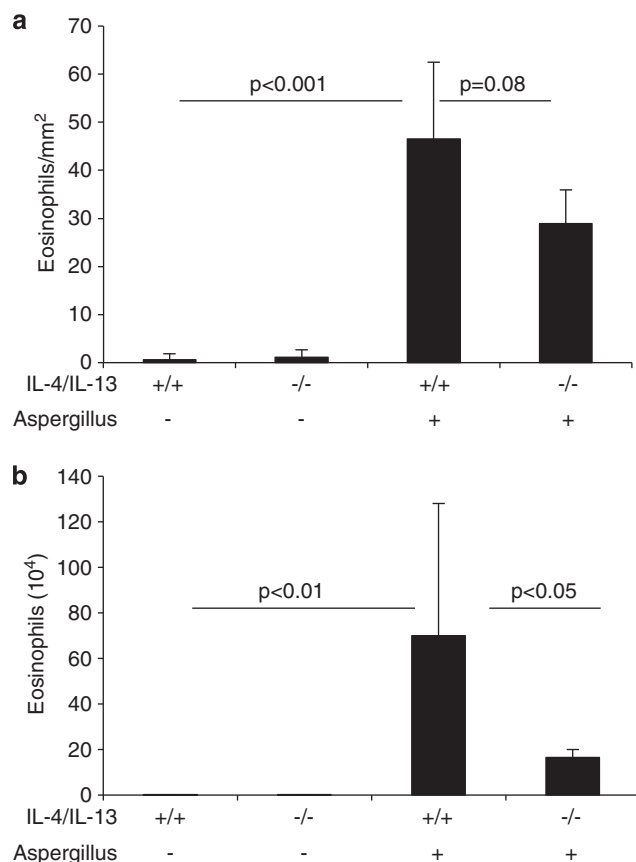


Figure 4 *Aspergillus*-induced eosinophilia in IL-4/IL-13 double gene-targeted mice. The level of eosinophils in the esophagus and lung of wild-type and IL-4/IL-13 gene-targeted mice were analyzed following intranasal nine doses of *Aspergillus* antigen challenge in 3 weeks of allergen challenge regime. Wild-type (+/+) or IL-4/IL-13 double gene-deficient (-/-) mice were challenged and eosinophils were determined by anti-MBP staining in the esophagus (a), and in the lung fluid (b) by differential counting of cells in BALF. The results are expressed as mean $\pm$ s.d. ($n=9$ mice per group).

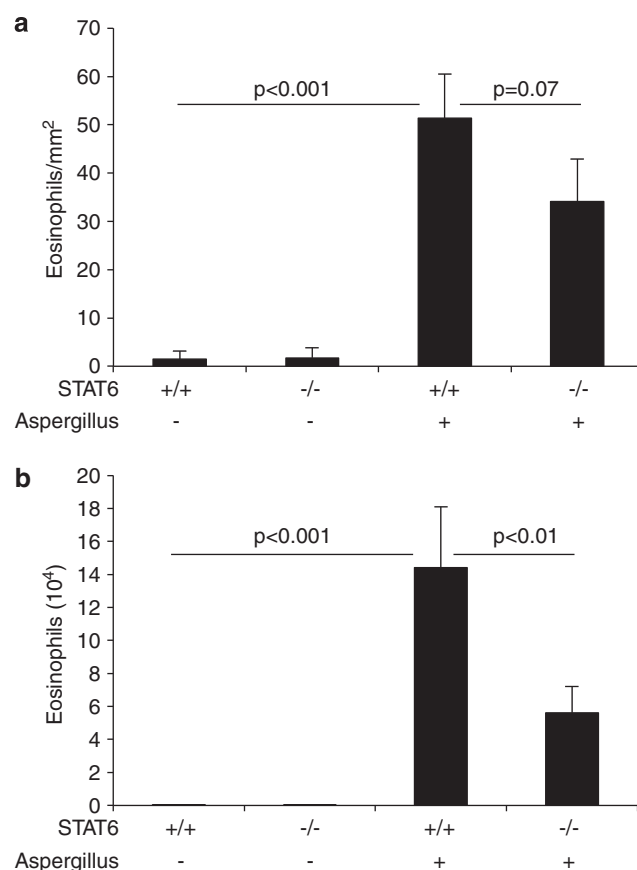


Figure 5 *Aspergillus*-induced eosinophilia in STAT6 gene-targeted mice. The level of eosinophils in the esophagus and lung of wild-type and STAT6 gene-targeted mice were analyzed following intranasal nine doses of *Aspergillus* antigen challenge in 3 weeks of allergen challenge regime. Wild-type (+/+) or STAT6-deficient (-/-) mice were challenged and eosinophils were determined by anti-MBP staining in the esophagus (a), and in the lung fluid (b) by differential counting of cells in BALF. The results are expressed as mean $\pm$ s.d. ($n=9$ mice per group).

eosinophilia was found to be dependent on IL-13. Our present data indicate that two different mechanisms are operational in IL-13-delivered lung eosinophilia and in allergen-induced EoE. We believe that the earlier reported coexpression of IL-5 and IL-13, including eosinophil-active chemokine eotaxin-3 in EoE patients³⁵ may be a co-incidence, and IL-13 may not be a major contributor in promoting human EoE. The earlier *in vitro* studies also show that IL-13 induces IL-5 and eosinophil-active chemokines eotaxin-1, -2 and -3 in the esophageal epithelial cells,³⁷ which also indicates that IL-13 induced IL-5 and eotaxins may be the primary cause of promoting esophageal eosinophilia. Furthermore, It has been also shown that IL-5 primes eosinophils to respond to chemoattractants and to induce eosinophil adhesion molecules' expression and activation.⁵¹ Taken together, all these previous reports and our present data suggest that IL-13-induced eosinophil accumulation in the esophagus is eotaxin and IL-5-mediated, and not a direct response of IL-13 that has been previously thought and reported.^{25,28,52} IL-13 is a pleiotropic cytokine and has been implicated in a variety of diseases. A number of reports indicate that IL-13 levels are increased in patients suffering from chronic asthma and EoE, and affect a variety of immune cell-mediated functions in several allergic diseases. Therefore, the presented experimental data indicate that IL-13 may not be critical in EoE pathogenesis.

This has been further confirmed by challenging the IL-13, both IL-4/IL-13 and STAT6 gene-deficient mice to the *Aspergillus* extract that IL-13 signaling is not required in promoting allergen-induced experimental EoE. These studies are important because a clinical report demonstrating that levels of IL-4-secreting T cells in the esophageal lesions along with IL-13 is elevated in patients with EoE.³⁸ Both IL-4 and IL-13 are related cytokines that share a common signal transduction mechanism involving the IL-4 receptor alpha chain and STAT6.¹⁶ Our experimentation showed that neither IL-4 receptor alpha chain-deficient mice (data not shown) nor IL-4/IL-13 double gene-deficient mice or the STAT6 gene-deficient mice were protected from allergen-induced EoE. Of note, both IL-4 and IL-13 are regulated by STAT6, and it is noteworthy that antigen-induced lung inflammation is largely dependent upon these signaling molecules. This we further confirmed by showing that *Aspergillus*-challenged IL-4/IL-13 double deficient and STAT6-deficient mice still had higher lung eosinophils compared with saline-challenged wild-type mice. In addition, we show that transcript expression levels of eosinophil-active cytokine IL-5 and chemokine eotaxin-1 are comparable in the allergen-challenged lung and esophagus of wild-type, STAT6 gene-deficient and IL-4/IL-13 double gene-deficient mice. However, the eotaxin-2 transcript expression was significantly reduced in the lungs along with partial reduction of lung eosinophilia in IL-4/IL-13

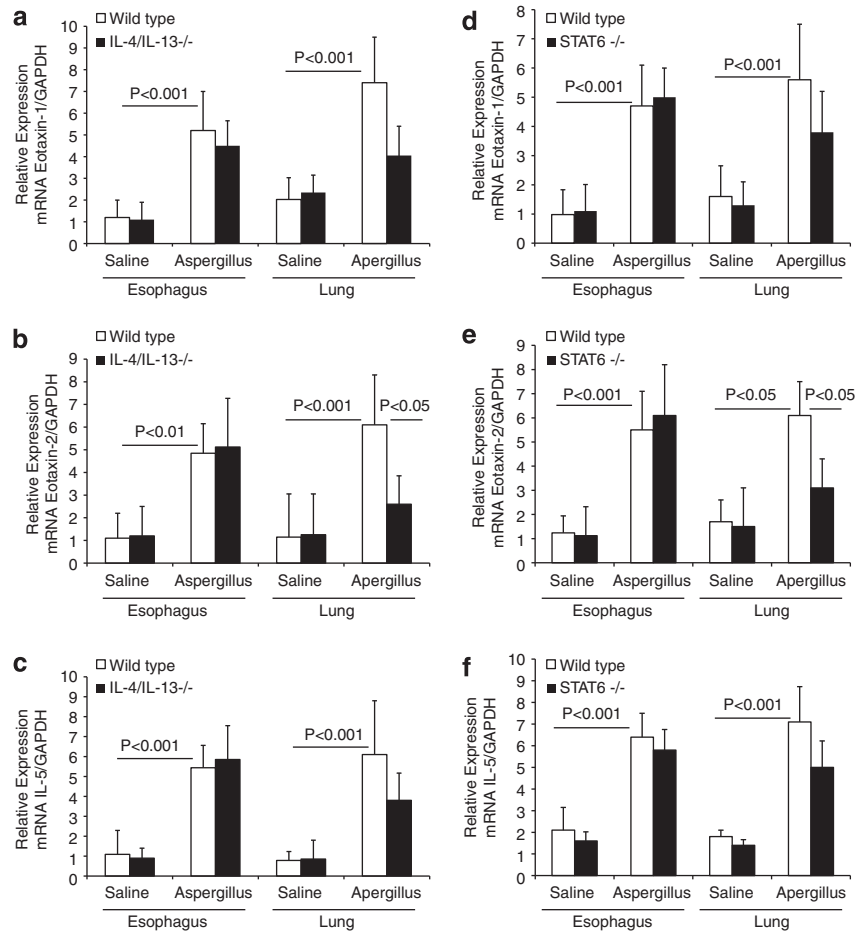


Figure 6 *Aspergillus*-induced mRNA expression of *eotaxin-1*, *eotaxin-2* and *IL-5* in the lung and esophagus. The quantitative real-time PCR analyses of *eotaxin-1*, *eotaxin-2*, and *IL-5* mRNA levels following saline- and *Aspergillus*-challenged in wild-type and IL-4/IL-13 double gene-deficient mice are shown (a–c) and wild-type and STAT6 gene-deficient mice are shown (d–f). The data are expressed as mean $\pm$ s.d., $n = 8$ mice per group.

double gene-deficient and STAT6 gene-deficient mice; but no reduction was observed in the esophageal eosinophilia or transcript expression. Therefore, it might be possible that *eotaxin-2* may have a role in partial eosinophil reduction in the lung, and not in the esophagus. These presented transcript expression data further indicate that two very different mechanisms are operational in the recruitment of eosinophils in lung and esophagus. We provide the first evidence that allergen-induced EoE is not dependent upon classic Th2 cytokine, IL-13 signaling, and draws attention to the importance of IL-5 (not IL-13) in disease pathogenesis. We clearly demonstrated that allergen-challenged IL-13 gene-deficient mice not only show comparable level of eosinophils, but also show similar levels of mast cells, intra-epithelial esophageal eosinophils and submucosal collagen in experimental EoE. These classical symptoms observed in IL-13 gene-deficient mice and wild-type mice following allergen challenges are the characteristic features of experimental and human EoE.^{40,53,54} These presented data further confirm the earlier published reports that IL-5 is critical in EoE, and esophageal remodeling in EoE is largely associated with the local expression of IL-5.^{40,55}

In summary, our present investigations dissect the cellular and molecular mechanisms involved in eosinophil accumulation in the esophagus, and demonstrate that allergen-induced eosinophilic esophageal disorder is independent of IL-4, IL-13 and its signaling factor STAT6, and further confirm the significance of our most recent

findings that allergen-induced EoE is STAT6-independent.⁵⁶ Taken together, these investigations indicate that IL-13-induced esophageal eosinophilia may be due to the induction of eotaxins, and IL-5 and IL-13 have no direct role in allergen-induced eosinophilic esophageal disorder. We conclude our study by stating that the role of IL-13 should be carefully interpreted in promoting EoE or suggesting any therapeutic interventions based on IL-13 in allergen-induced eosinophilic esophageal disorders.

METHODS

Mice

Eight week-old BALB/c mice, STAT6-deficient and IL-5 deficient, with matched control mice (Jackson Laboratory, Bar Harbor, ME, USA), IL-13 deficient, IL-4/IL-13 double deficient and their littermate BALB/c controls (originally obtained from A MacKenzie of Medical Research Council Laboratory of Molecular Biology, Cambridge, UK) were kept and used at CCHMC, Cincinnati. Partial tissue analysis was performed at Case Western University, Cleveland, OH, USA. All procedures were performed in accordance with the ethical guidelines in the Guide for the Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committee-approved protocol.

Induction of experimental EoE in mice

Experimental EoE was induced in mice, following an established protocol described previously.⁴⁰ In brief, mice were lightly anesthetized with isoflurane inhalation (methoxy-fluorane; Pittman-Moore, Mundelein, IL, USA) and

100 µg (50 µl) of *Aspergillus fumigatus* (Greer Laboratories, Lenoir, NC, USA) or 50 µl of normal saline alone was applied to the nares using a micropipette, with the mouse held in the supine position. After instillation, mice were held upright until alert. After three treatments per week for 3 weeks, mice were killed between 18 and 20 h after the last intranasal challenge. Additionally, we also challenged the mice with three doses of recombinant IL-13 10 µg per 40 µl (or 40 µl saline) at an interval of 24 h and the eosinophil level in the esophagus was determined 24 h after the last IL-13 delivery.

BALF collection and analysis

The mice were euthanized by CO₂ inhalation. Immediately thereafter a midline neck incision was made and the trachea was cannulated. The lungs were lavaged three times with 1.0 ml phosphate-buffered saline, containing 1% fetal calf serum and 0.5 mM EDTA. The recovered BALF was centrifuged at 400 × g for 5 min at 4 °C, and resuspended in 200 µl phosphate-buffered saline, containing 1% fetal calf serum and 0.5 mM EDTA. Lysis of red blood cells was carried out utilizing RBC lysis buffer (Sigma, St Louis, MO, USA), according to the manufacturer's recommendations. Total cell numbers were counted with a hemacytometer. Cytospin preparations of 5 × 10⁴ cells were stained with Giemsa-Diff-Quick (Dade Diagnostics of PR, Inc, Aguada, PR, USA), and differential cell counts were determined for eosinophils percent and absolute numbers.

Eosinophil analysis in the esophagus

The esophagus of adult mice was fixed in 4% paraformaldehyde in phosphate buffer pH 7.4, embedded in paraffin, cut into 5-µm sections, fixed to positively charged slides and immunostained with antiserum against mouse eosinophil major basic protein (anti-MBP), a kind gift of Dr James and Dr Nancy Lee (Mayo Clinic, Scottsdale, AZ, USA), as described.⁴⁰ In brief, endogenous peroxidase in the tissues was quenched with 0.3% hydrogen peroxide in methanol, followed by nonspecific protein blocking with normal goat serum. Tissue sections were then incubated with rabbit anti-MBP (1:16 000) overnight at 4 °C, followed by 1:200 dilution of biotinylated goat anti-rabbit IgG secondary antibody and avidin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 30 min each. These slides were further developed with nickel diaminobenzidine-cobalt chloride solution to form a black precipitate, and counterstained with nuclear fast red. Negative controls include replacing the primary antibody with normal rabbit serum to check endogenous biotin and peroxidase activity. Quantification of the immunoreactive cells was carried out by using a video-assistant-integrated computer software program Image Pro software analyzer (Media Cybernetics, Warrendale, PA, USA). The eosinophil levels are expressed as cells mm⁻².

Mast cell analysis

The 5-µm esophageal paraffin tissue sections were de-paraffinized, stained with a chloroacetate staining and detected by performing histological analysis using light microscopy. The pink color mast cells were quantified by counting the stained cells in each tissue section with the assistance of digital morphometry using the Image Pro software analyzer (Media Cybernetics) and expressed as mast cells mm⁻² tissue area as described earlier.^{25,36}

Tissue collagen staining

Esophageal tissue samples were fixed with 4% paraformaldehyde, embedded in paraffin, cut into 5-µm sections and fixed to positively charged slides. Collagen staining was then performed on the tissue sections by Masson's trichrome (Poly Scientific R&D Corp, Bay Shore, NY, USA) method for the detection of collagen fibers according to the manufacturer's recommendations. The collagen thickness was measured using the Image Pro software analyzer (Media Cybernetics) and is expressed as collagen thickness in µm.

Real-time PCR analysis

The RNA samples (500 ng) were subjected to reverse transcription using iScript reverse transcriptase (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The cytokine and chemokine mRNA levels were

Table 1 Primers used to quantitate IL-5, eotaxin-1 and eotaxin-2 mRNA levels

Genes	Sense and antisense primer sequence
mIL-5	5'-TCCCATGAGCACAGTGGTGAAAG-3' 5'-CACAGTACCCCCACGGACAGTTT-3'
mEotaxin-1	5'-GGCTCACCCAGGCTCCATCC-3' 5'-TTTGGTCCAGGTGCTTTGTGG-3'
mEotaxin-2	5'-CTCCTTCTCCTGGTAGCTGC-3' 5'-GTGATGAAGATGACCCCTGCCTT-3'
GAPDH	5'-TGGAAATCCCATCACCATCT-3' 5'-GTCTTCTGGGTGGCAGTGAT-3'

quantified by real-time PCR using the LightCycler instrument and LightCycler FastStart DNA master SYBR green I as a ready-to-use reaction mix (Roche, Indianapolis, IN, USA). Results were then normalized using previously published human *GAPDH* primers^{28,37,52,55} to amplified mouse *GAPDH* from the same cDNA mix and expressed as fold induction compared with the controls. cDNA was amplified using the primers described earlier. The primers used to perform qPCR are listed in Table 1.

Statistical analysis

Data are expressed as mean ± s.d. Statistical significance comparing different set of mice was determined by unpaired InStat GraphPad *t*-test (GraphPad, La Jolla, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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