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

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### Abstract

Several studies have shown that IL-13 is induced in the esophageal biopsies of 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.

### Introduction

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 impactions in adolescents and adults.<sup>1–4</sup> Both pediatric and adult EoE patients develop fibrosis and other anatomical complications including

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esophageal strictures.<sup>4-12</sup> EoE is now considered a global health problem for children in multiple developed and developing countries over the last decade.<sup>1, 5, 12-19</sup> 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.<sup>20, 21</sup> Interestingly, IL-13 appears to be particularly important since it is produced in high quantities by Th2-cells and regulates multiple characteristics of allergic diseases.<sup>22</sup> The levels of elevated IL-13 is an important regulator of a number of allergic diseases including asthma,<sup>23</sup> eosinophilic esophagitis,<sup>24-28</sup> atopic dermatitis,<sup>29-31</sup> and allergic rhinitis.<sup>32, 33</sup> IL-13 share a common receptor subunit, with the IL-4 R $\alpha$ , and signals through the signal transducer and activator of transcription (STAT)6.<sup>34</sup> Th2 cells produce the cytokine IL-5, which is specific for the growth and survival of eosinophils. We demonstrated earlier that IL-5 is over-expressed in the esophagus of patients with EoE<sup>35</sup> and systemic over-expression of IL-5 (via pharmacological or transgenic approaches) promotes EoE in mice.<sup>36</sup> It has been previously shown that *in vitro* IL-13 activates esophageal epithelial cells and induces eosinophil chemokines, eotaxin-1, -2 and -3.<sup>37</sup> Additionally, it has also been shown that IL-13 induces IL-5 that may be responsible for IL-13 induced tissue eosinophilia.<sup>26</sup> Therefore, it is important to understand the role of IL-13 in promoting esophageal eosinophilia, whether IL-13 directly acts and promote 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.<sup>38, 39</sup> Further, we also previously showed that allergen challenge promotes IL-5 mediated experimental asthma and EoE in mice.<sup>40</sup> 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.<sup>40</sup> Importantly, over-expression of IL-13, by transgenic approaches, induces multiple features of EoE, including eosinophilia, collagen deposition and reduced lumen circumference.<sup>25, 41</sup> Therefore, it is 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 signal transducer and activator of transcription (STAT)6 gene-deficient mice. The data presented in this manuscript establish that IL-13 intranasal delivery promote IL-5 dependent esophageal eosinophilia; however, IL-13 signalling 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, 10mg of recombinant IL-13 was delivered intranasally to the wild-type mice 3 times on alternate days. Mice received 3 doses of recombinant IL-13 10 $\mu$ g/ 40 $\mu$ l (or 40 $\mu$ l saline) separated by 24 hours and the eosinophil level in the esophagus was determined 24 hours 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  $24.3 \pm 4.6$  and  $1.1 \pm 1.6/\text{mm}^2$  following 3 intranasal IL-13 treatment (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.<sup>26</sup> 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 following 3 doses of 10 mg IL-13 treatment on alternate days were  $23.1 \pm 8.4$  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  $1.1 \pm 1.4$  and  $0.9 \pm 1.2/\text{mm}^2$ , respectively. For comparison, eosinophil levels in the BALF of wild-type and IL-5 gene targeted mice following 3 doses of 10 mg intranasal IL-13 were  $10.6 \pm 2.7 \times 10^4$  and  $0.57 \pm 0.48 \times 10^4/\text{lung}$  (mean  $\pm$  S.D.,  $n = 6-8$ ;  $P < 0.001$ ), whereas, saline treated wild type and IL-5 gene-deficient mice had  $0.005 \pm 0.01 \times 10^4/\text{lung}$  and  $0.002 \pm 0.004 \times 10^4/\text{Lung}$ , respectively (Figure 1B). Collectively, these results establish an essential role for IL-5 in IL-13-induced esophageal eosinophilia. This data is similar to our earlier **reported intratracheal delivered IL-13** findings;<sup>25</sup> 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 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  $43.8 \pm 9.1$  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  $1.3 \pm 1.8$  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  $23.2 \pm 3.2 \times 10^4/\text{lung}$  (mean  $\pm$  S.D.,  $n = 8$ ) compared with  $6.6 \pm 2.8 \times 10^4/\text{lung}$  in IL-13 gene deficient mice (mean  $\pm$  S.D.,  $n = 8$ ;  $P < 0.01$ ); whereas, saline treated wild type and IL-13 gene-deficient mice had  $0.01 \pm 0.01 \times 10^4$  and  $0.01 \pm 0.01 \times 10^4/\text{lung}$  (mean  $\pm$  S.D.,  $n = 8$ ), respectively (Figure 2A, B). Furthermore, most of the EoE characteristics such as the induction of intraepithelial eosinophilia (Figure 3 A, B), esophageal mast cells (Figure 3 C, D) and lamina propria collagen (Figure 3 E, F) was 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 (Figure 3,G, 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-4R $\alpha$  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.

### **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.<sup>42, 43</sup> 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  $46.5 \pm 16.4$  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  $0.6 \pm 1.3$  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  $63.9 \pm 51.5 \times 10^4/\text{lung}$  (mean  $\pm$  S.D.,  $n = 8$ ) compared with  $12.8 \pm 0.5 \times 10^4/\text{lung}$  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  $0.01 \pm 0.1 \times 10^4/\text{lung}$  (mean  $\pm$  S.D.,  $n = 8$ ), respectively (Figure 4 A, B). Collectively, these results establish that allergen-induced EoE is not dependent on IL-4/IL-13.

### **Aspergillus-induced eosinophilic esophagitis 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.<sup>44</sup> 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  $51.4 \pm 9.1$  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  $1.4 \pm 1.7$  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  $14.4 \pm 3.7 \times 10^4/\text{lung}$  (mean  $\pm$  S.D.,  $n = 8$ ) compared with  $5.6 \pm 1.6 \times 10^4/\text{lung}$  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  $0.01 \pm 0.01 \times 10^4/\text{lung}$  (mean  $\pm$  S.D.,  $n = 8$ ), respectively (Figure 5 A–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 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 to the saline-challenged wild type mice (Figure 6 A–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 (Figure 6 A, C) and allergen challenged wild type and *STAT6* gene-deficient mice (Figure 6 D, 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 to their respective wild type mice (Figure 2 B, E). These data indicates 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.<sup>2, 45–49</sup> Recent clinical studies have suggested that the prevalence of these disorders, especially EoE, is increasing frequency.<sup>48, 50</sup> 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.<sup>40</sup> 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*.<sup>25, 41</sup> However, the significance of *IL-13* in allergen-induced EoE was not earlier proven. Notably, EoE is an allergen-induced esophageal disorder.<sup>20, 21</sup> Therefore, the present study was designed to tested 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* induce eosinophilia in the lung.<sup>26</sup> 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 characteristics feature like comparable intraepithelial eosinophils, induced and lamina propria collagen accumulation following allergen challenge. In contrast, the allergen-induced lung eosinophilia was found dependent to *IL-13*. Our present data indicates that two different mechanism is 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 patients<sup>35</sup> may be a co-occurrence 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 esophageal epithelial cells,<sup>37</sup> 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 molecule expression and activation.<sup>51</sup> 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.<sup>25, 28, 52</sup> IL-13 is a pleiotropic cytokine and has been implicated in the 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 cells mediated functions in several allergic diseases. Therefore, the presented experimental data indicates 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.<sup>38</sup> 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.<sup>16</sup> 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 is 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 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 indicates 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 showing comparable level of eosinophils but also show similar levels of mast cells, intraepithelial 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.<sup>40, 53, 54</sup> 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.<sup>40, 55</sup>

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 to 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.<sup>56</sup> 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 has 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.

## Materials and Methods

### Mice

Eight week-old BALB/c mice, STAT6 deficient and IL-5 deficient with matched control mice (Jackson Laboratory, Bar Harbor, ME), 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, United Kingdom) were kept and used at CCHMC, Cincinnati. Partial tissue analysis was performed at Case Western University, Cleveland, Ohio. 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.<sup>40</sup> In brief, mice were lightly anesthetized with isoflurane inhalation (methoxy-fluorane; Pittman-Moore, Mundelein, IL) and 100 µg (50 µl) of *Aspergillus fumigatus* (Greer Laboratories, Lenoir, NC) 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 three weeks, mice were sacrificed between 18 and 20 hours after the last intranasal challenge. Additionally, we also challenged the mice with 3 doses of recombinant IL-13 10µg/ 40µl (or 40µl saline) separated by 24 hours and the eosinophil level in the esophagus was determined 24 hours after the last IL-13 delivery.

### Bronchoalveolar lavage fluid (BALF) collection and analysis

The mice were euthanized by CO<sub>2</sub> inhalation. Immediately thereafter a midline neck incision was made and the trachea was cannulated. The lungs were lavaged three times with 1.0 ml PBS containing 1% FCS and 0.5 mM EDTA. The recovered BALF was centrifuged at 400× g for 5 minutes at 4°C, and resuspended in 200 µl PBS containing 1% FCS and 0.5 mM EDTA. Lysis of red blood cells was carried out utilizing RBC lysis buffer (Sigma, St. Louis, MO) according to the manufacturer's recommendations. Total cell numbers were counted with a hemacytometer. Cytospin preparations of 5 × 10<sup>4</sup> cells were stained with Giemsa-Diff-Quick (Dade Diagnostics of P.R., Inc., Aguada, PR) 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 5mm sections, fixed to positive charged slides, and immunostained with antiserum against mouse eosinophil major basic protein (anti-MBP), a

kind gift of Drs. James and Nancy Lee (Mayo Clinic, Scottsdale, AZ), as described.<sup>40</sup> 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) for 30 minutes 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). The eosinophil levels are expressed as cells/mm<sup>2</sup>.

### **Mast cell analysis**

The 5- $\mu$ m 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 stained cells in each tissue section with the assistance of digital morphometry using the Image Pro software analyzer (Media Cybernetics, Warrendale, PA) and expressed as mast cells/mm<sup>2</sup> tissue area as described earlier.<sup>25, 36</sup>

### **Tissue collagen staining**

Esophageal tissue samples were fixed with 4% paraformaldehyde, embedded in paraffin, cut into 5 $\mu$ m sections, and fixed to positively charge slides. Collagen staining was then performed on the tissue sections by Masson's trichrome (Poly Scientific R&D Corp.) 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, Warrendale, PA) and is expressed as collagen thickness in  $\mu$ m.

### **Real-time polymerase chain reaction (PCR) analysis**

The RNA samples (500 ng) were subjected to reverse transcription using iScript reverse transcriptase (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The cytokine and chemokine mRNA levels were 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). Results were then normalized using previously published **human GAPDH primers**<sup>28,37,52,55</sup> to amplified mouse GAPDH from the same cDNA mix and expressed as fold induction compared to the controls. cDNA were amplified using the primers described earlier. The primers used to perform qPCR are listed in Table 1.

### **Statistical analysis**

Data are expressed as mean  $\pm$  Standard Deviation (S.D.). Statistical significance comparing different set of mice was determined by unpaired InStat GraphPad t-test.



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## Abbreviations used in this paper

<b>BALF</b>	bronchoalveolar lavage fluid
<b>EoE</b>	eosinophilic esophagitis
<b>MBP</b>	major basic protein
<b>STAT</b>	signal-transducer-and-activator-of-transcription

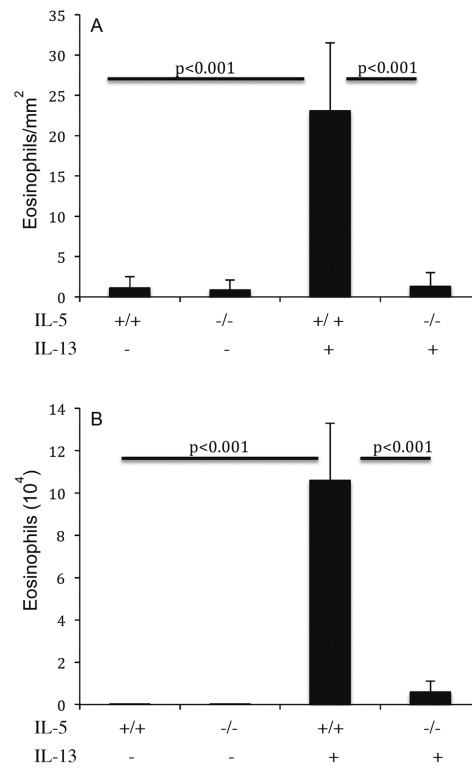
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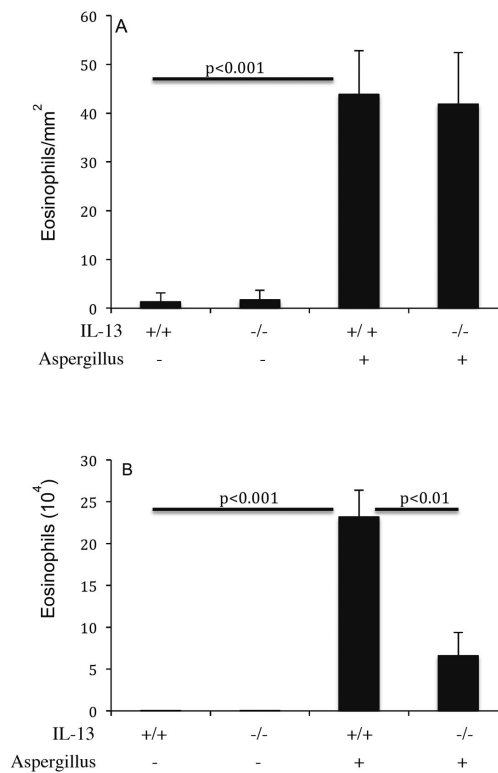
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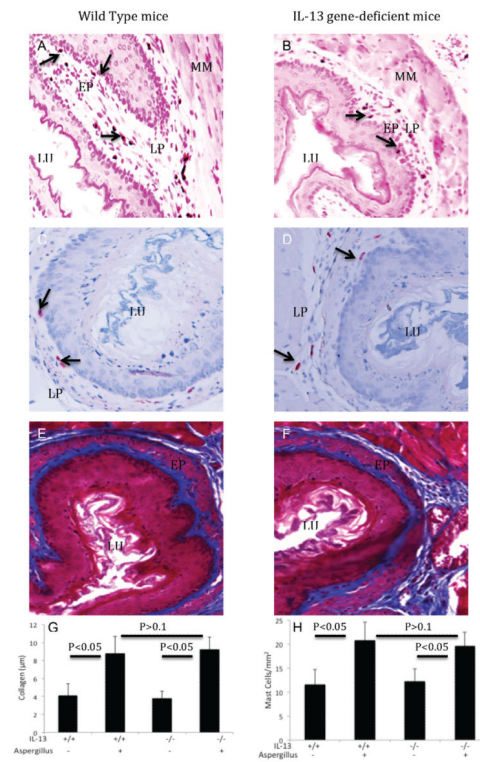


**Figure 1. IL-13-induced esophageal eosinophilia in wild type and IL-5 gene-targeted mice**  
 The level of eosinophils in the esophagus and lung of wild type and IL-5 gene targeted mice were analyzed following intranasal 10 mg or rIL-13 challenge. Wild-type (+/+) or IL-5-deficient (-/-) mice were challenged 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/group).



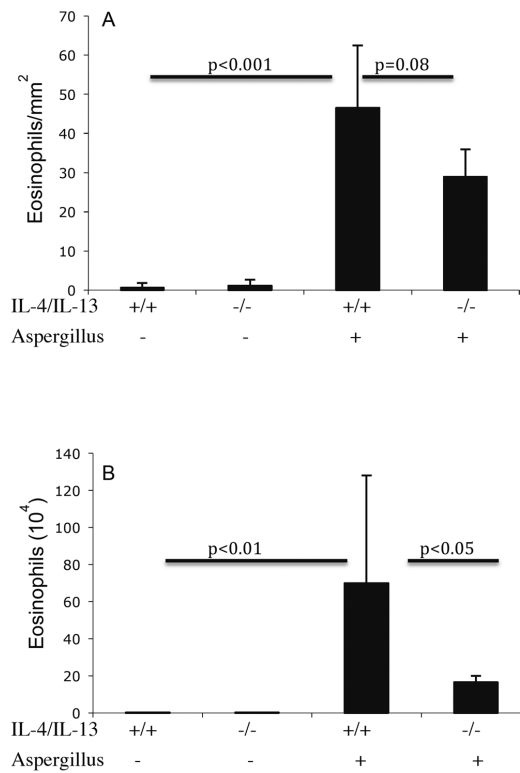
**Figure 2. Aspergillus-induced eosinophilia in IL-13 gene-targeted mice**

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/group).



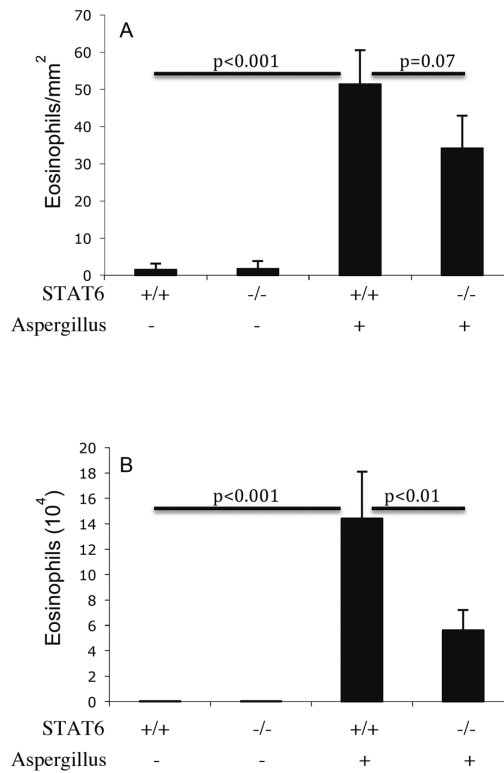
**Figure 3. Detection of intraepithelial eosinophils, mast cells and collagen in IL-13 gene-deficient mice following Aspergillus-induced EoE**

The EoE characteristics 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 intraepithelial eosinophils (A, B, original magnification 200 $\times$ ), mast cells (C, D, original magnification 200 $\times$ ) and lamina propria and epithelial mucos collagen accumulation (E, F, original magnification 400 $\times$ ). Arrows indicates intraepithelial eosinophils or lamina propria mast cells in the respective photomicrographs. The quantitation of collagen thickness and mast cells numbers in wild type and IL-13 gene-deficient mice are shown (G, H). The results are expressed as mean  $\pm$  S.D. (n = 8 mice/group). EP-Epithelium, LP-Lamina propria, LU-Lumen, MM-Muscular Mucosa,



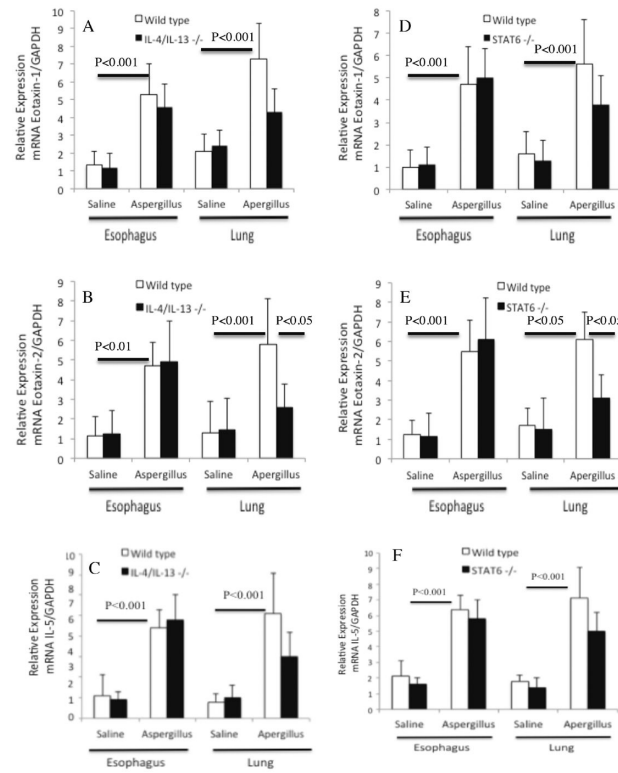
**Figure 4. Aspergillus-induced eosinophilia in IL-4/IL-13 double gene-targeted mice**  
 The level of eosinophils in the esophagus and lung of wild type and IL-4/IL-13 gene targeted mice were analyzed following intranasal nine doses of Aspergillus antigen challenge in 3 weeks of allergen challenge regime. 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/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/group).



**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/group.

**Table 1**

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

Genes	Sense and anti-sense primer sequence
mIL-5	5'-TCCCATGAGCACAGTGGTGAAAG 5'-CACAGTACCCCCACGGACAGTTT
mEotaxin-1	5'-GGCTCACCCAGGCTCCATCC 5'-TTTTGGTCCAGGTGCTTTGTGG
mEotaxin-2	5'-CTCCTTCTCCTGGTAGCCTGC 5'-GTGATGAAGATGACCCCTGCCTT
GAPDH	5'-TGGAAATCCCATCACCATCT 5'GTCCTTCTGGGTGGCAGTGAT

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