Elevated levels of interleukin (IL)-18 have been reported in a number of allergic diseases. We recently reported that IL-18 in the blood and IL-18Rα mRNA in the oesophagus are induced during human eosinophilic oesophagitis (EoE). Additionally, we earlier showed that invariant natural killer T (iNKT) cells are critical to EoE pathogenesis; however, the mechanism of iNKT cell activation in EoE is not well understood. Therefore, the current study focused on the hypothesis that allergen-induced IL-18 may have an important role in iNKT cell-mediated EoE pathogenesis. We first validated the human EoE findings of IL-18 in experimental EoE by examining blood levels of IL-18 and oesophageal IL-18Rα mRNA levels in aeroallergen- and food allergen-induced experimental mouse models of EoE. We demonstrate that blood IL-18 protein and oesophageal IL-18Rα mRNA are induced in the mouse model of EoE and that IL-18Rα is expressed by iNKT cells in the oesophagus. Intranasal delivery of rIL-18 induced both mast cells and eosinophilic inflammation in the oesophagus in a time- and dose-dependent manner. To establish the significance of IL-18 in EoE pathogenesis, we examined DOX-inducible rTAT-CC10-IL-18 bitransgenic mice that induce IL-18 protein expression in the oesophagus. Our analysis indicated that induction of IL-18 in these mice resulted in the development of many of the characteristics of EoE, including oesophageal intraepithelial eosinophilia, increased mast cells, oesophageal remodelling and fibrosis. The current study provides evidence that IL-18 may induce iNKT cell activation to release the eosinophil-activating cytokine IL-5, as IL-5-deficient mice and iNKT cell-deficient (CD1d null) mice do not induce EoE in response to intranasal IL-18 challenge. Taken together, these findings provide evidence that allergen-induced IL-18 has a significant role in promoting IL-5- and iNKT-dependent EoE pathogenesis.
eosinophil and mast cell accumulation in the oesophagus. Additionally, we found that IL-5-deficient mice and iNKT cell-deficient (CD1d-deficient) mice are protected from EoE induction following intranasal delivery of IL-18. Taken together, our current findings provide insight into the role of allergen-induced IL-18 in earlier reported IL-5- and iNKT cell-mediated EoE pathogenesis.

RESULTS
Blood IL-18 and oesophageal IL-18Rα expression levels are induced in experimental EoE
Our previously reported microarray data showed increased IL-18R transcript levels in EoE patients compared with normal individuals.8 Therefore, we sought to understand the role of IL-18 in EoE...
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Interleukin-18 transgene overexpression promotes eosinophilic and mast cell inflammation in the oesophagus
To validate that IL-18 overexpression induces eosinophils and mast cells in the oesophagus, we obtained DOX-inducible rtTA-CC10-IL-18 bitransgenic mice from Dr Jack Elias, MD, PhD, Yale University. The DOX-inducible IL-18 transgenic mice showed no significant increase in IL-18 mRNA; however, significantly increased IL-18 protein levels were observed in the oesophagus compared with the no-DOX mice. Control mice treated with intratracheal saline showed a baseline level of oesophageal mast cells at each dose of saline (Figure 2b).

IL-18 overexpression in mice promotes oesophageal fibrosis
Chronic tissue eosinophilia and mast cell inflammation are implicated in promoting oesophageal remodelling and fibrosis in EoE. Therefore, we were next interested in establishing that IL-18-induced eosinophilic and mast cell inflammation promote oesophageal fibrosis in DOX-inducible IL-18 bitransgenic mice. Accordingly, oesophageal tissue sections of no-DOX and DOX-exposed IL-18 transgenic mice were stained with Masson’s trichrome for collagen accumulation in the eosophagus following Aspergillus-induced experimental EoE (Figures 1g–i; n = 3 experiment).

Intranasal delivery of rIL-18-induced oesophageal eosinophilia and mast cell inflammation in mice
We next tested the hypothesis that IL-18 intranasal delivery promotes oesophageal eosinophilia in mice. Accordingly, mice were intranasally challenged with 5 doses of IL-18 on alternate days using the techniques established to induce experimental EoE.24–26 Mice that received 1–5 doses of recombinant (r) IL-18 or saline on alternate days developed oesophageal eosinophilia 24 hours after each administration of IL-18 at two different doses (5 and 10 μg). In fact, the mice developed detectable oesophageal eosinophilia even after one treatment, and the severity increased with subsequent treatments before reaching a plateau between the fourth and fifth dose. Control mice treated with intranasal saline did not have significant levels of oesophageal eosinophils (Figure 2a). As expected, IL-18 intranasal delivery also induced pulmonary eosinophilia in the same mice (data not shown). By contrast, intranasal delivery of IL-18 had no effect on eosinophil levels in the stomach (42.8 ± 28.2 vs 57.9 ± 19.4) eosinophils per mm² in IL-18- versus saline-treated mice (mean ± s.d., P = 0.8)). These results establish that intranasal delivery of IL-18 promotes concentration-dependent oesophageal eosinophilia. A number of clinical reports have indicated that mast cells are also induced in EoE. Therefore, we next examined oesophageal mast cells in mice that received 1–5 doses of 5 or 10 μg rIL-18 (or saline) on alternate days. IL-18-treated mice developed a detectable increase in oesophageal mast cells even after 1 dose, and their numbers continued to increase with subsequent doses. The control mice treated with intratracheal saline showed a baseline level of oesophageal mast cells at each dose of saline (Figure 2b).

IL-18 transgene overexpression promotes eosinophilic and mast cell inflammation in the oesophagus
To validate that IL-18 overexpression induces eosinophils and mast cells in the oesophagus, we obtained DOX-inducible rtTA-CC10-IL-18 bitransgenic mice from Dr Jack Elias, MD, PhD, Yale University. The DOX-inducible IL-18 transgenic mice showed no significant increase in IL-18 mRNA; however, significantly increased IL-18 protein levels were observed in the oesophagus compared with the no-DOX mice following 3 weeks of exposure (Figures 3a and b). Notably, the DOX-inducible IL-18 bitransgenic mice are driven by a lung-specific Clara cell promoter (CC-10). The no-DOX and DOX-exposed IL-18 transgenic mice were evaluated for EoE pathogenesis. The DOX-treated mice showed most of the EoE characteristics, including eosinophil accumulation in the lamina propria and epithelial mucosa (intraepithelial eosinophilia) along with extracellular eosinophilic granules in the oesophageal mucosa. The no-DOX mice showed few baseline eosinophils and only in the oesophageal lamina propria (Figures 3c and f, mice = 12 per group). The number of eosinophils in the oesophagus of DOX-exposed IL-18 bitransgenic mice was 72.6 ± 1.1 mm² compared with 4.2 ± 1.1 mm² (mean ± s.d., mice = 12 per group, P < 0.001) in no-DOX IL-18 bitransgenic mice (Figure 3g). Interestingly, the IL-18 bitransgenic mice treated with DOX for 3 weeks also developed mast cell inflammation in the oesophagus. Most of the mast cells in DOX-exposed mice were detected in the lamina propria and some in the muscularis mucosa (Figure 4b and data not shown). No-DOX bitransgenic mice showed a comparatively low number of baseline mast cells in the oesophageal lamina propria (Figure 4a). For example, the number of mast cells in the oesophagus of DOX-exposed IL-18 bitransgenic mice was 32.4 ± 10.3 mm² compared with 11.6 ± 3.8 mm² in no-DOX exposed IL-18 bitransgenic mice (Figure 4c). The data are expressed as the mean ± s.d., mice = 12 per group).
Figure 3 IL-18 transgene overexpression promotes EoE in mice. rtTA-CC10-IL-18 bitransgenic mice were exposed to DOX food or normal food (No DOX) and oesophageal IL-18 mRNA expression (a) and IL-18 protein (b) levels were quantitative by RT-PCR and ELISA analysis, respectively. Anti-MBP immunostained oesophageal eosinophilia in the lamina propria as well as in the epithelial mucosa (c–f). Intraepithelial eosinophils were detected in the oesophagus of DOX-exposed tissue sections (e, f). Representative photomicrographs from 12 mice tissue sections are shown in both low (×100) and high (×400) of original magnification. Quantitation of eosinophils in the oesophageal section of DOX- and no-DOX-exposed rtTA-CC10-IL-18 bitransgenic mice (g). The data show the means ± s.d. pooled from three experiments (4 mice per group per treatment per experiments; total mice = 12 per group). *P<0.001. EP, Epithelium; LP, Lamina propria; MS, Muscularis Mucosa; LU, Lumen.
epithelial mucosa and lamina propria to detect oesophageal fibrosis. Oesophageal tissue sections from no-DOX and DOX-exposed IL-18 bitransgenic mice were analysed by trichrome staining for collagen accumulation in the epithelial mucosa and for fibrosis in the lamina propria. Analysis of oesophageal sections demonstrated normal epithelial and muscularis mucosa and lamina propria with organised, thin sub-epithelial trichrome material in the no-DOX mice (Figure 5a). By contrast, the oesophagus of DOX-exposed IL-18 bitransgenic mice showed collagen induction and expansion of connective tissue in the oesophageal epithelial mucosa, lamina propria and muscularis mucosa (Figure 5b). Interestingly, we observed that most DOX-treated IL-18 transgenic mice had shorter lumens compared with no-DOX mice. A semi-quantitative analysis performed using digital morphometry (Luminera Corporation, Infinity Analyze 6.1.0) indicated ~threefold increase of lamina propria collagen thickness in DOX-exposed IL-18 bitransgenic mice. (Figure 5c). The lamina propria collagen thickness in the oesophagus of DOX-exposed IL-18 transgenic mice was 12.2 ± 4.2 mm² compared with 3.3 ± 1.3 mm² in no-DOX IL-18 transgenic mice. The data are expressed as the mean ± s.d., mice = 12 per group.

**IL-5- and CD1d-deficient mice are protected from oesophageal eosinophilia induction following intranasal IL-18 delivery**

IL-5 and iNKT cells have been shown to be critical in promoting experimental EoE, as IL-18-activated iNKT cells produce eosinophil activating cytokines, including IL-5, in an antigen-independent manner. Most recently, we have shown that a non-immortalised human iNKT cell line exposed to IL-18 in vitro produces IL-5 and IL-13. Therefore, to understand the mechanism of IL-18-induced experimental EoE, wild-type, IL-5-deficient and CD1d-deficient mice were given intranasal saline or IL-18 (5 doses of 5 μg) on alternate days, and oesophageal eosinophilia and mast cells were examined in the oesophagus as described earlier. Both IL-5-deficient and iNKT-deficient mice were protected from IL-18-induced oesophageal eosinophilia compared with wild-type mice, which developed significant oesophageal eosinophilia (Figures 6a and c). However, a comparable number of oesophageal mast cells was found in both IL-5- and CD1d-deficient mice compared with wild type (Figures 6b and d). The data are expressed as the means ± s.d., mice = 12 per group, P < 0.001.

**DISCUSSION**

Oesophageal eosinophilia occurs in a variety of clinical disorders, including gastroesophageal reflux disease (GERD), eosinophilic gastroenteritis and EoE. A number of clinical studies suggest that these disorders (especially EoE) are occurring with increasing frequency, mostly in well-developed countries. Our earlier studies indicated that iNKT cells and IL-18Rα transcripts were induced in EoE patients, and IL-5 plays a critical role in the initiation and progression of the disease. IL-18-activated iNKT cells release eosinophil activating cytokines, such as IL-5 and IL-13. In addition, IL-18 induction has been reported in a number of food allergen-
induced diseases, such as food allergy, coeliac disease, asthma and allergic colitis. Therefore, the current study was designed to understand the molecular processes involved in IL-5- and iNKT cell-mediated EoE pathogenesis. iNKT cell accumulation and activation have been reported in human EoE; but the mechanism that accounts for the activation of iNKT cells in human EoE is not yet well understood. The current data show that blood IL-18 and oesophageal IL-18Rα mRNA are increased following experimental EoE induction by repeated intranasal exposure of aeroallergen or food (peanut) allergen, which is consistent with our previous findings in human EoE. Our data also indicate that IL-18Rα+ cells accumulate in the oesophagus following EoE induction, and we confirm that these induced IL-18Rα+ cells are iNKT cells. Furthermore, we show that intranasal IL-18 delivery in mice promotes oesophageal eosinophils and mast cells in a dose- and time-dependent manner. Interestingly, both eosinophilia and mast cell accumulation in the oesophagus are basic characteristics of EoE. We speculate that IL-18 induced in the oesophagus might be due to overproduction of IL-18 protein in the lung, which is then swallowed by the mice.

To establish the mechanism by which IL-18 promotes EoE, we showed that iNKT cell-deficient (CD1d−/−) mice are protected from the induction of EoE following exposure to rIL-18. CD1d null mice were examined because earlier reports indicated that iNKT cells are induced and critical in EoE. iNKT activation promotes the eosinophil-activating cytokines IL-5 and IL-13, which are implicated in the induction and progression of both experimental and human EoE. Notably, earlier studies showed that IL-18 is capable of activating iNKT cells without T-cell receptor engagement, and activation by IL-18 induces Th2 cytokines from T cells or mast cells. Additionally, the combination of IL-18 and IL-2 in naïve mice induces IgE production. A similar protection in oesophageal eosinophilia was observed in IL-5-deficient mice following rIL-18 challenge. These data established that IL-18 may activate iNKT cells to release IL-5 and promote EoE. iNKT cell activation has also been shown by the Aspergillus-derived glycosphingolipid asperamide B. Therefore, it is possible that asperamide B may activate iNKT cells via CD1d in Aspergillus-induced iNKT cell-mediated EoE. However, our current data indicate that increased IL-18 in human and experimental EoE may be sufficient to activate iNKT cells, as most food-allergic patients

**Figure 5** Oesophageal fibrosis is induced in IL-18-overexpressed mice. The characteristic features of EoE oesophageal fibrosis were examined by Masson’s trichrome staining for the accumulation of collagen in the oesophageal tissue sections. A significant increase of collagen in the lamina propria, epithelial mucosa and muscularis mucosa of 3 weeks DOX-exposed mice were detected compared with the baseline collagen in the lamina propria of no-DOX mice (a, b, magnification ×40). Morphometric analysis of lamina propria collagen thickness per mm² is shown (c). The data show the means ± s.d. pooled from three experiments, (4 mice per group per treatment per experiments; total mice = 12 per group). EP, Epithelium; LP, Lamina propria; MS, Muscularis Mucosa; LU, Lumen.

**Table 1** Summary of the molecular mechanisms involved in IL-18-mediated EoE pathogenesis.

| Mechanism | Summary |
|-----------|---------|
| IL-18 | Promotes EoE by activating iNKT cells, which release IL-5 and promote EoE. |
| iNKT cells | Critical in EoE, induced by IL-18 |
| Aspergillus-derived glycosphingolipid asperamide B | Activates iNKT cells via CD1d |

**Table 2** Comparison of the characteristics of experimental and human EoE.

| Characteristic | Experimental EoE | Human EoE |
|---------------|-----------------|-----------|
| Oesophageal eosinophilia | Yes | Yes |
| Oesophageal inflammatory infiltrate | Yes | Yes |
| Oesophageal fibrosis | Yes | Yes |
| Oesophageal muscle hypertrophy | Yes | Yes |

**Table 3** Comparison of the characteristics of experimental and human EoE.

| Characteristic | Experimental EoE | Human EoE |
|---------------|-----------------|-----------|
| Oesophageal eosinophilia | Yes | Yes |
| Oesophageal inflammatory infiltrate | Yes | Yes |
| Oesophageal fibrosis | Yes | Yes |
| Oesophageal muscle hypertrophy | Yes | Yes |

**Table 4** Comparison of the characteristics of experimental and human EoE.

| Characteristic | Experimental EoE | Human EoE |
|---------------|-----------------|-----------|
| Oesophageal eosinophilia | Yes | Yes |
| Oesophageal inflammatory infiltrate | Yes | Yes |
| Oesophageal fibrosis | Yes | Yes |
| Oesophageal muscle hypertrophy | Yes | Yes |
have induced IL-18. More recently, we showed that a human iNKT cell line exposed to IL-18 produces the eosinophil-activating cytokines IL-5 and IL-13. Furthermore, it is well established that B cells also express IL-18R and are a source of IgE. B cells and IgE are enhanced in EoE patients; therefore, it is possible that IL-18-mediated mast cell degranulation may also have an important role in the pathogenesis of EoE. Mast cells have long been considered to play a significant role in the pathophysiology of allergic diseases through their ability to release a host of pleiotropic autacoid mediators, proteases and cytokines in response to activation by both IgE-dependent and diverse non-immunologic stimuli. They are the source of several neutral proteases, such as tryptase and chymase, which interact with many cells and potentially contribute to tissue remodelling. It has been shown that tryptase and chymase are induced in human EoE; therefore, it is possible that these IL-18-induced IgE/mast cell responses may influence oesophageal remodelling in the mucosa, leading to oesophageal fibrosis.

Taken together, we demonstrated that mice with experimental EoE have increased levels of blood IL-18 and IL-18R mRNA in the oesophagus. We showed that iNKT cells induced in the oesophagus express IL-18R in a murine model of EoE. IL-18 overexpression by pharmacological delivery or transgene insertion promotes EoE. Mechanistically, we showed that iNKT cell-deficient and IL-5-deficient mice are protected from the induction of EoE, which is in accordance with the earlier report that IL-18 stimulates iNKT cells to produce abundant eosinophil-activating cytokines, including IL-5 and IL-13. In conclusion, the current study provides an improved understanding of the significance of IL15 and IL-15-responsive CD1d-restricted iNKT cells in EoE. Additionally, we suggest a novel role for IL-18 in promoting iNKT-mediated allergen-induced EoE.

METHODS

Mice
Specific pathogen-free BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). CD1d gene-deficient and IL-5 gene-deficient Balb/c background was obtained from Drs Jochen Mattner, MD and Marc Rothenberg, MD, PhD, (Cincinnati Children’s Hospital Medical Center, Cincinnati, OH, USA). The CC10-IL-18 mice were obtained from the laboratory of Dr Elias, MD, PhD, (Yale University). The mice were maintained in a pathogen-free barrier facility. All the experiments were performed on age- and gender-matched, 6-8-week-old mice. The Institutional Animal Care and Use Committee (IACUC) approved the animal protocol is accordance to National Institute of Health guidelines. Therefore, all the experiments performed are according to animal ethics rules and regulation.
Experimental EoE
A mouse model of allergic EoE was established using methods described previously. In brief, mice were lightly anaesthetised with isoflurane (Iso-Flo; Abbott Laboratories, North Chicago, IL, USA), and 100 μg of Aspergillus fumigatus (Greer Laboratories, Lenoir, NC, USA) in 50 μl normal saline or 50 μl of normal saline alone was given intranasal using a micropipette with the mouse held in the supine position. In addition, we also induced experimental EoE by sensitizing the mice at 0 and 14 days with peanut extract 200 μg and 1 mg Alum and then challenged them with 100 μg peanut on day 21, 23 and 25. After three treatments per week for 3 weeks, the mice were killed between 20 and 24 h after the last intranasal allergen or saline challenge.

Quantitative PCR
The RNA samples (300 ng) were subjected to reverse transcription using Bioretscript reverse transcriptase (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. IL-18Rα mRNA from the oesophageal RNA was quantified by real-time PCR using IQ5 (Bio-Rad). Results were then normalised to human or mouse GAPDH amplified from the same cDNA mix and expressed as relative gene expression. CDNAs were amplified using the primers sequence for IL-18Rα F 5′-CTGCCGTGACGTTCCTCCTGGAAG-3′; R 5′-TGCTCCTGGAAACACGGT TCTCGAAAGA-3′ and GAPDH F 5′-TGGAAAATC CATCCKACCATC-3′; R 5′-GTCTTCTGGATGGCACTGA T-3′.

Isolation of oesophageal cells and the analysis of IL-18Rα expression on inNKT cells by flow cytometric analyses
The isolated oesophageal cells were stained with cell surface molecule-specific antibodies for flow cytometer analyses. The oesophageal cells were isolated as per the protocol described earlier. The following reagents were used for specific antigen analysis: anti-CD4, and isotype controls obtained from eBiosciences (San Diego, CA, USA). The mouse CD1d-tetramer was obtained from the tetramer core facility of the National Institute of Allergy and Infectious Diseases. FcR block (anti-CD16/CD32) was added to all surface-staining mixtures. 7-Amino actinomycin D was used to exclude dead cells. The cells were incubated with antibodies to specific antigens at 4°C for 45 min followed by two washes. The flow cytometric analyses were performed using a FACS Calibur (BD Biosciences, San Jose, CA, USA) and analyzed using flowjo software (Treestar, Ashland, OR, USA).

Oesophageal eosinophil analyses
The 5-μm oesophageal paraffin tissue sections were immunostained with antiseraum against mouse eosinophil major basic protein (anti-MBP, a kind gift of Drs James and Nancy Lee, Mayo Clinic, Scottsdale, AZ, USA) as described. In brief, endogenous peroxide in the tissue was quenched with 0.3% hydrogen peroxide in methanol followed by non-specific protein blocking with normal rabbit serum. Tissue sections were then incubated with rat anti-MBP (1:2000) overnight at 4°C, followed by 1:200 dilution of biotinylated anti-rat IgG secondary antibody and avidin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 30 min each. The slides were further developed with nickel dibenzenobenzidine-cobalt chloride solution to form a black precipitate, and counterstained with nuclear fast red. Negative controls included replacing the primary antibody with normal rabbit serum.

Oesophageal mast cell analysis
The 5-μm oesophageal paraffin tissue sections were de-paraffinised and stained with hexazonised new fuchsin (Sigma Aldrich, St Louis, MO, USA) with 4% sodium nitrate in naphthol-AS-D chloroacetate (Sigma Aldrich) and phosphate buffered saline solution for 30 min and counterstained with haematoxylin. The histological analysis was performed using light microscopy.

Quantification of tissue eosinophils and mast cells
Eosinophils and mast cells were quantified by counting the anti-MBP-positive cells in the epithelial mucosa and lamina propria of the oesophagus. Eosinophil numbers and area of the each oesophageal tissue section was measured and calculated with the assistance of digital morphometric analysis (Luminera Corporation, (Hicksville, NY, USA) Infinity Analyze 6.1.0) and expressed as eosinophils per mm² as described previously. Further, the same software was used to measure epithelial cell layer thickness.

Collagen staining
Oesophageal tissue samples were fixed with 4% paraformaldehyde, embedded in paraffin, cut into 5-μm sections and fixed to positively charged slides. The collagen staining was performed on the tissue sections using Mason’s trichrome (Poly Scientific R&D Corporation, Bay Shore, NY, USA) for the detection of collagen fibbers according to the manufacturer’s recommendations.

Statistical analysis
For all cell counts, stained slides were analysed randomly and in a blinded manner. The nonparametric Mann–Whitney U-test was employed for comparison of data between two groups, and Kruskal–Wallis for comparison of more than two groups. Parametric data were compared using paired t-tests or analysis of variance. Values are reported as mean ± s.d. P-values<0.05 were considered statistically significant.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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