

Elevated IL-33 expression is associated with pediatric eosinophilic esophagitis, and exogenous IL-33 promotes eosinophilic esophagitis development in mice

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Submitted 24 August 2015; accepted in final form 8 October 2015

Judd LM, Heine RG, Menheniott TR, Buzzelli J, O'Brien-Simpson N, Pavlic D, O'Connor L, Al Gazali K, Hamilton O, Scurr M, Collison AM, Mattes J, Allen KJ, Giraud AS. Elevated IL-33 expression is associated with pediatric eosinophilic esophagitis, and exogenous IL-33 promotes eosinophilic esophagitis development in mice. *Am J Physiol Gastrointest Liver Physiol* 310: G13–G25, 2016. First published October 29, 2015; doi:10.1152/ajpgi.00290.2015.—We tested whether the T helper (Th) type 2 (Th2) cell agonist and allergenic ligand IL-33 was associated with eosinophilic esophagitis (EoE) development in a pediatric cohort and whether IL-33 protein could induce disease symptoms in mice. Biopsies from EoE patients or controls were used to measure IL-33 mRNA and protein expression. Increased expression of IL-33 mRNA was found in the esophageal mucosa in EoE. IL-33 protein was detected in cells negative for CD45, mast cells, and epithelial cell markers near blood vessels. Circulating levels of IL-33 were not increased. The time course for IL-33 gene expression was quantified in an established *Aspergillus fumigatus* allergen mouse model of EoE. Because IL-33 induction was transient in this model and chronicity of IL-33 expression has been demonstrated in humans, naive mice were treated with recombinant IL-33 for 1 wk and esophageal pathology was evaluated. IL-33 application produced changes consistent with phenotypically early EoE, including transmural eosinophilia, mucosal hyperproliferation, and upregulation of eosinophilic genes and chemokines. Th2 cytokines, including IL-13, along with innate lymphoid cell group 2, Th1/17, and M2 macrophage marker genes, were increased after IL-33 application. IL-33-induced eosinophilia was ablated in IL-13 null mice. In addition, IL-33 induced a profound inhibition of the regulatory T cell gene signature. We conclude that IL-33 gene expression is associated with pediatric EoE development and that application of recombinant protein in mice phenocopies the early clinical phase of the human disease in an IL-13-dependent manner. IL-33 inhibition of esophageal regulatory T cell function may induce loss of antigenic tolerance, thereby providing a mechanistic rationale for EoE development.

eosinophilic esophagitis; IL-33; food allergy; Th2

EOSINOPHILIC ESOPHAGITIS (EoE) is a chronic inflammatory condition of the esophagus, distinct from gastroesophageal reflux disease, the etiology of which is associated with allergy to food

and airborne antigens and the development of atopy (1). It is most common in young children, with prevalence rates of ~1 in 10,000 (2); however, the incidence is increasing, along with the global increase in food allergies (37), especially in settings of low chronic infection or parasitic disease.

EoE is characterized immunologically by a strongly enhanced mucosal T helper (Th) type 2 (Th2) cell cytokine response marked by high levels of IL-13 and IL-5 (3, 27). As the name implies, the esophageal mucosa is infiltrated by large numbers of eosinophils, as well as mast cells, which are attracted by migratory factors such as chemokine (C-C motif) ligand (CCL) 26 (eotaxin 3) and play a key role in promoting the inflammation and fibrosis that characterize chronic EoE pathogenesis. Candidate disease genes derived from genome-wide association studies include CCL26 (4) and thymic stromal lymphopoietin (TSLP) (34), a Th2 cytokine regulator that has increased expression and is associated with basophilia in mouse models and clinical cases of EoE in humans (32).

Although the literature on the epidemiology and clinical management of EoE has grown in recent years, an understanding of the pathological drivers and immune response repertoire is incomplete. For instance, the role of innate lymphoid cell group 2 (ILC2), which activates Th2 cytokines in gut and respiratory mucosae, in EoE induction is not well described, although TSLP (20) and IL-25/IL-17E (14) can activate ILC2. This also raises the following question: Is IL-33, the third established driver of Th2/ILC2, associated with EoE initiation and progression.

IL-33 is an IL-1 cytokine family ligand that signals by binding a receptor complex consisting of IL-1 receptor type 1 and IL-1 receptor-like 1 (ST2) (36). IL-33 can induce IL-4, IL-5, IL-9, and IL-13 (37), key cytokines in EoE pathogenesis, which together promote eosinophil, mast cell, and basophil activation and migration and all of which express ST2. IL-33 is produced constitutively in numerous cell types including gut epithelial cells (17), and its induction is associated with increased serum IgE (19) in the presence of antigen and atopic disease, including asthma, eczema (22), and rhinitis (13). Since IL-33 has many of the key attributes required to produce the cellular and immunological outcomes observed in EoE pathol-

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ogy and has been detected by immunohistochemistry in human esophageal epithelium in active EoE (38), we first investigated whether its gene expression is elevated in esophageal biopsies of a clinically diagnosed pediatric EoE cohort compared with disease-free controls. Having determined a positive association between IL-33 and established EoE, we then evaluated whether endogenous IL-33 is induced in the *Aspergillus fumigatus* mouse model of EoE and whether exogenously applied IL-33 induces EoE disease symptoms in the esophagus. The latter treatment for 1 wk produced an EoE phenotype, including eosinophilia and hyperproliferation, likely driven by key regulatory genes that are, coincidentally, upregulated in the early stages of EoE in humans. Since IL-33 strongly induced IL-13 in the mouse esophagus, the effect of genetic ablation of IL-13 on IL-33-induced inflammation was also evaluated. In addition, we quantified the expression of genes associated with the innate and adaptive immune response in mouse esophagus after exogenous IL-33 treatment and compared this profile with the untreated esophagus. The predominant induction of Th2 cytokines, M2 macrophages, and ILC2 suggests a mechanism by which IL-33 may promote esophageal inflammation secondarily to its potential effect on suppressing regulatory T (Treg) cells and inducing the loss of immune tolerance.

METHODS

Clinical

Biopsy selection and collection. Fourteen pediatric patients undergoing upper gastrointestinal endoscopy for suspected EoE were recruited into the study. Patients were subsequently divided into normal controls ($n = 5$) and those with confirmed EoE ($n = 9$). Subjects' history and consent were obtained, and exclusions were made as follows: comorbidities, including inflammation elsewhere in the gastrointestinal tract, and past or current treatment for EoE, including steroid use or dietary intervention. During gastroscopy, grasp biopsies were collected from the upper and lower esophagus, as well as the gastric body, antrum, and duodenum. In addition, two biopsies were taken from the lower esophagus: one was immediately frozen in liquid nitrogen for mRNA analysis, and the other was fixed in 4% paraformaldehyde in PBS for histology. Blood (2.5 ml) was collected from patients and placed into a serum gel tube. Within 2 h the blood was centrifuged (5,300 rpm, 5 min, 4°C) to isolate serum, which was stored at -80°C. All clinical samples were analyzed by independent pathologists, and diagnosis of EoE was made on the basis of ≥ 15 eosinophils per high-power field ($\times 400$ magnification in esophageal biopsies and absence of increased eosinophilia in stomach and duodenum). An absence of inflammation throughout the upper gastrointestinal tract was confirmed for all control samples. This study was approved by the Royal Children's Hospital Human Ethics Committee (HREC 30211A).

IL-33 ELISA. Serum samples (50 μ l) were analyzed using a proprietary human IL-33 ELISA (eBioscience). The procedure was performed according to the manufacturer's instructions.

Mouse

***A. fumigatus* model of EoE.** The *A. fumigatus* model of EoE was developed (10) and validated (26) as previously described. Allergen [*A. fumigatus* extract (Greer Laboratories), 100 μ g in 50 μ l of saline, $n = 6$ –8] or saline alone was given intranasally to lightly anesthetized BALB/c mice thrice weekly for 3 wk, and esophageal tissue was taken for quantification of IL-33 by quantitative PCR at 1 day, 1 wk, and 3 wk. EoE symptoms, including eosinophilia and mast cell expansion, develop progressively after 1 wk of dosing (31). Studies were ap-

Table 1. Sequence of primers used for quantitative RT-PCR

Gene	Primer	
	Forward (5'-3')	Reverse (5'-3')
hIL33	GGCTGAGAATTACCATACAAGG	AGTGTTTTTTCAGATGGGATGA
hIL5	GAAAGAGACCTTGGCACTGCTT	TCCTCAGAGTCTCATTTGGCTATC
hIL13	GATTCTGCCCGCACAAGGT	CGATTTTGGTGTCTCGGACAT
hCCL11	GCGACTAGAGAGCTACAGGAGA	GGTCTTTAAGATCAGAGCTTTCT
hCCL26	TCGCTCCTGAGTCTCCACCTT	CTGTATTGGAAGCAGCAGGTCTT
hCCL24	TGATGACCATAGTAACCGACCTT	CACAGAGCCCGTAGGGATGA
hTGF β 1	CGCGTGCTAATGGTGGAAA	TGTGTGTACTCTGCTGAACCTTG
hSMAD7	TCCAGATACCCGATGGATTTC	CCCTGTTTCAGCGGAGGAA
hIFN γ	CCAGGACCCATATGTAAGAAG	TCATGTCTTCTGTATGGTCTCC
hIL4	ACCGAGTTGACCGTAACAGACA	GTAGAACTGCCGGAGCAGAGT
hTSLP	AGTGGGACCAAAAGTACCGAGTT	GGGGTGGGATTGAAGGTTAG
hIL10	AACCAAGACCCAGACATCAAGG	CTCACTCATGGCTTTGTAGATGC
hIL1 β	ATGACCTGAGCACCTTCTTTCC	CTGTGTGCTCCATATCCTGTCC
hIL13	GTGCCAAGATCTGTGTCTCTCC	TTACAGAGGCCATGCAATATCC
mIL5	TTGACAAGCAATGAGACGATGAG	GCCCTGAAAGATTCTCCAA
mIFN γ	TGCCACGGCACAGTCATT	CCAGTTCCTCCAGATATCCAAGA
mCCL11	CAATGCCACCCCTGGTCTACA	AGGGAAGGGTGTGAGAAATG
mCCL5	CCAATCTTGCAGTCGTGTTTGT	CTTCTCTGGCTTGGCACACA
mCCL12	TGGACAGATGCGGTGAG	GGCTGCTGTGATTTCTCTGT
mTGF β 1	GGTGTGCTCTATGCCATCAA	CCTCTCCGATTACCGCTGAT
mTSLP	GGCTACCCCTGAACTGAGAGAAA	GGTTTGATTTCAGGCAGATGTTTT
mMBP	TCAGTGGCCCCAAGGAAGAG	CGGGCAAACTGACTGAGC
mCCL24	TCTCTGCAGGCGGACCTT	CCCACCTTAAGACAAGAACCCTA
mIL1 β	CAGGCAGTATCACTCATTGTGG	GTGCAGTTGTCTAATGGGAACG
mIL1 α	AACCCATGATCTGGAAGAGACC	TGGTGCTGAGATAGTGTGTTGCC
mIL33	TATGAGTCTCCCTGTCTCTGCAA	CTCATGTTCCACCATCACCTTCTTC
mIL9	CGTCCCAACTGATGATTGTACC	TGCATCTCTGTCTTCTGGAACG
mIL4	GAGACTCTTTCGGGCTTTTTCG	TCTTTCAGTGATGTGGACTTGA
mCD19	AGGTCATTGCAAGGTCAGCAGTG	GGCGTCACTTTGAAGAATCTCCT
mIgE	ACACTGGAGATGCCAGATC	GGAGCACCGTTTGTATACAGGTC
mPrss34	CGGTTCCCTCATCCATCCT	CTTTCGGCTCACACAGTGA
mFollistatin	GAGGAGGATGTGAACGACAATAC	GGGTTTATTCTTCTGTTTCATTCC
mIGF1	TGCTCTTCAGTTTCGTGTG	ACATCTCCAGTCTCCTCAG
mGATA3	CGAGACATAGAGAGCTACGCAAT	ACCTGAGTAGCAAGGAGGCTAG
mAmphiregulin	CTATCTTTGTCTGCCATCATC	CAACTTTTACCTTTGCAATTGCC
mIL13	TGCCCCAAGATCTGTGTCTCTCC	TTACAGAGGCCATGCAATATCC
mIL5	TTGACAAGCAATGAGACGATGAG	GCCCTGAAAGATTCTCCAA
mIFN γ	TGCCACGGCACAGTCATT	CCAGTTCCTCCAGATATCCAAGA
mIL17A	CTGTGTCTCTGATGCTGTGTC	ACATCTGGAGGAAGTCCCTGG
mST2	CACCAGATCATTCACAGTTG	CCACTTCCATTGTGTGGTTGTA
mArg1	AAAGCTGGTCTGTGAAAA	ACAGACCGTGGGTTCTTCAC
mYml	TCTGGTGAAGGAAATCGCTAAA	GCAGCCTTGAATGTCTTTCTC

h, Human; m, murine; CCL, chemokine (C-C) motif ligand; TGF, transforming growth factor; TSLP, thymic stromal lymphopoietin; MBP, major basic protein; Prss, protease, serine 34; IGF1, insulin-like growth factor-1; Arg1, arginase-1.

proved by the Animal Care and Ethics Committee of the University of Newcastle.

Cytokine treatment. A cohort ($n \geq 10$) of 10- to 12-wk-old wild-type (WT, C57BL/6) mice were injected intraperitoneally once daily with 1 μ g of recombinant human IL-33 (Shenandoah) or saline for 7 days. At 1 h prior to culling, the mice were given an additional dose of IL-33. To test the importance of IL-13 signaling in IL-33-induced inflammation, 10- to 12-wk-old IL-13^{-/-} BALB/c mice or WT controls were treated as described above. These studies were approved by the Murdoch Childrens Research Institute Animal Ethics Committee (#A741).

Tissue preparation. The esophagus was removed from each mouse after anesthetic overdose. For histological examination, tissue was bisected sagittally, fixed flat overnight in 4% paraformaldehyde in PBS, and processed for embedding in paraffin blocks. For mRNA analysis, the whole esophagus was washed and then frozen in liquid nitrogen until extraction.

Quantitative RT-PCR

Quantitative RT-PCR primer sequences are given in Table 1.

Total RNA was harvested using TRIzol reagent (Life Technologies). RNA [1–3 μ g, $n = 5$ –9 samples/group for human EoE and

Table 2. Antibodies used for immunohistochemistry

Antibody	Raised In	Supplier	Dilution	Product No.
Anti-IL33	Mouse	Enzo Life Science	1:200	ALX-804-840-C100
Anti-Ki67	Rabbit	Abcam	1:100	Ab15580
Anti-mast cell chymase	Goat	Abcam	1:50	Ab111239
Anti-pan-cytokeratin	Rabbit	Abcam	1:250	Ab9377
Anti-CD45	Rabbit	Abcam	1:250	Ab40763

8–10 samples/treatment for mouse analyzed independently in 2 separate experiments ($n = 4$ –5), and data were pooled if qualitative changes were consistent between experiments] was reverse-transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Promega) and then primed with oligo(dT). Quantitative RT-PCR (qRT-PCR) primers were designed using Primer Express (Applied Biosystems; Table 1). SYBR Green chemistry, with β -actin (*A. fumigatus* mRNA analysis) or rL32 (all other mRNA analyses) as the internal reference gene, was used. qRT-PCR conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 15 s (AB7500, Applied Biosystems). Results were analyzed using sequence detector software, and relative fold differences were determined using the cycle threshold ($\Delta\Delta C_t$) method.

Histology and Immunohistochemistry

For histological analysis, 4- μ m paraffin sections were stained with hematoxylin and eosin, Alcian blue/periodic acid-Schiff (neutral and acidic mucins), or Sirius red (collagen). For immunohistochemical analysis, 4- μ m paraffin sections on 3-aminopropyltriethoxysilane-coated slides were subjected to immunohistochemistry. Antigen retrieval was carried out in 10 mM citric acid at 100°C for 30 min, and the samples were allowed to cool for 30 min. Details for primary antibodies are given in Table 2. Staining was completed with species-specific biotinylated secondary antibodies, avidin, and biotinylated horseradish peroxidase complex (Vector Laboratories), 3,3'-diaminobenzidine, and hematoxylin counterstaining. Immunofluorescent staining was performed with fluorescently conjugated species-specific secondary antibodies. Samples were mounted in ProLong Gold containing 4',6-diamidino-2-phenylindole counterstain (Invitrogen). Fluorescence images were captured using a laser scanning confocal microscope (TCS SP2, Leica) and additionally processed using LAS AF Lite software (Leica). For all staining reactions, a control was performed with secondary antibody alone. Representative images from each group are shown.

Quantitative Morphometry

All quantitative morphometry was performed by a blinded observer. At least three representative photographs per animal ($n \geq 5$) of histochemically or immunohistochemically stained sections were analyzed at $\times 400$ magnification, and stained cells or regions were counted manually over the entire section using an eyepiece-calibrated graticule or graduated viewing rectangles.

Statistical Analysis

Values are means $\pm$ SE. Statistical analysis was performed using one-way analysis of variance and the appropriate parametric or non-parametric statistical test using SigmaStat (Jandel Scientific, San Rafael, CA). $P \leq 0.05$ was considered statistically significant.

RESULTS

Clinical Cohort

Mean age was 5.32 ± 1.35 yr in the control group and 9.65 ± 1.71 yr in the EoE group. There was no statistical difference between group ages ($P = 0.112$, by *t*-test). Typically, 10–90 eosinophils per high-power field (42 ± 13) were observed in the upper esophagus and 0–87 (29 ± 11) in the lower esophagus of EoE patients compared with none in controls. Upper gastrointestinal disease symptoms were shown by all EoE patients for ≥ 2 yr, with the most common being food allergies, dysphagia, vomiting, regurgitation, food aversion, slow weight gain, asthma, and eczema. Biopsies were taken at initial diagnosis ($n = 5$ or 9, age range 5–16 yr) and during follow-up (2.4 ± 1.1 yr after diagnosis, $n = 4$ or 9, age range 6–16 yr). The control group commonly presented with regurgitation and recurrent abdominal pain.

Esophageal Biopsies From EoE Patients Have Increased Expression of Th2 Ligands, CCL26, and IL-33

It is well established that the esophageal mucosa mounts a strong Th2 response in EoE and that the eosinophil- and mast cell-inducing factor CCL26 (eotaxin 3) is also highly expressed and diagnostic for this condition. Since IL-33 can induce Th2 activation, we measured tissue gene (mRNA) expression for IL-33, as well as a range of EoE-related ligands, in control and EoE biopsies by quantitative PCR (Fig. 1A). As expected,

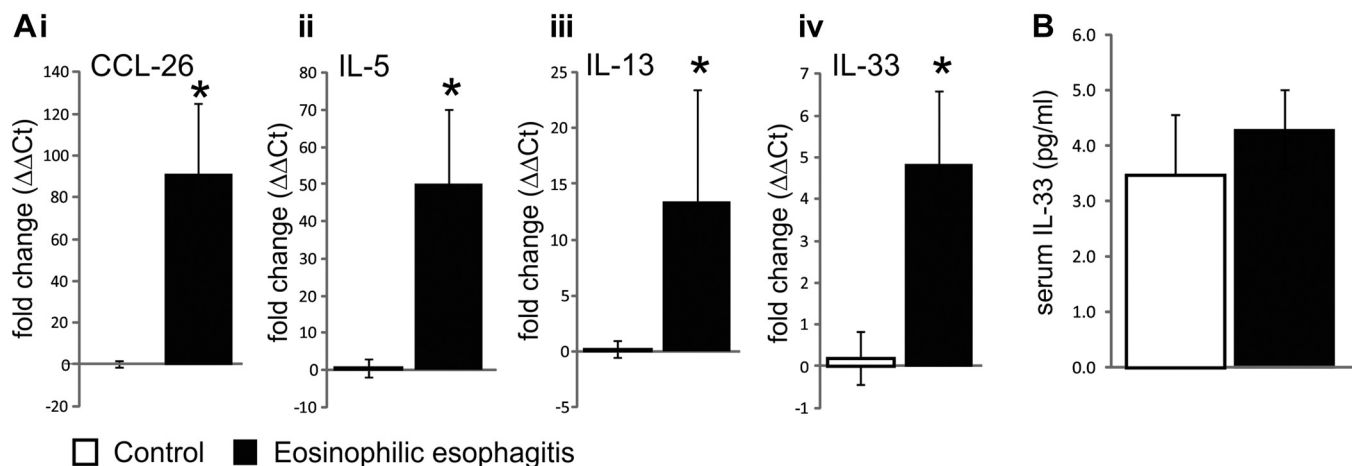
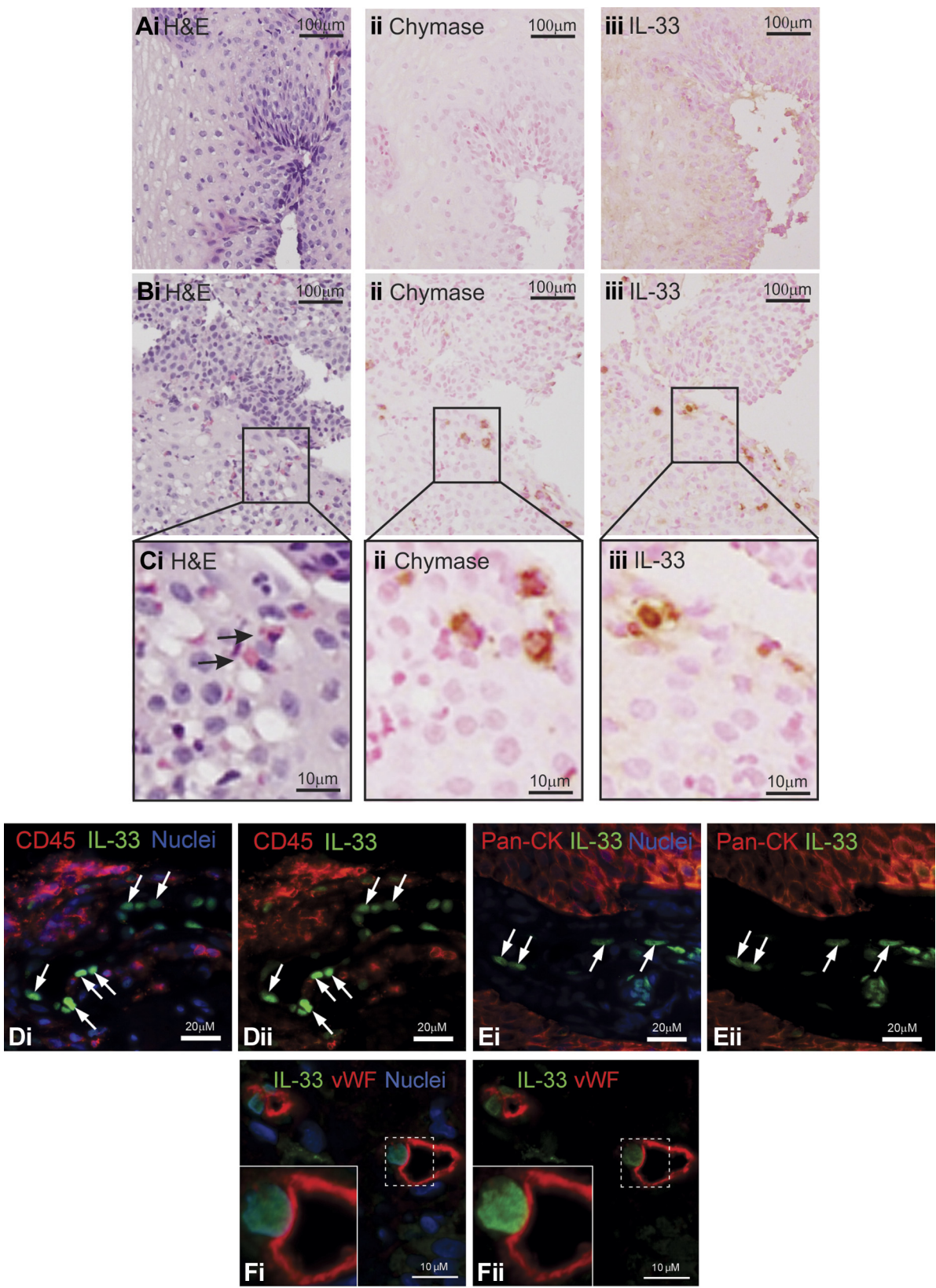


Fig. 1. Expression of T-helper (Th) cell type 2-related chemokines and cytokines is increased in pediatric eosinophilic esophagitis (EoE). RNA was extracted from esophageal biopsies from control subjects ($n = 5$) and EoE patients ($n = 9$). A: quantitative RT-PCR for chemokine (C-C motif) ligand (CCL) 26 (i), IL-5 (ii), IL-13 (iii), IL-33 (iv), and the housekeeping gene L32. Values (means $\pm$ SE) are expressed as fold change [by the cycle threshold ($\Delta\Delta C_t$) method]. * $P < 0.05$ vs. control. B: ELISA of serum IL-33 from control subjects and EoE patients. No significant difference was detected ($P > 0.05$).



CCL26 was the most upregulated gene, being increased 90-fold in EoE (Fig. 1Ai; $P = 0.04$), while the key Th2 ligands IL-5 and IL-13 were increased 50-fold (Fig. 1Aii; $P = 0.04$) and 13-fold (Fig. 1Aiii; $P = 0.04$), respectively. IL-33 gene expression was increased fivefold (Fig. 1Aiv; $P = 0.03$) in the same cohort, while the following genes were not changed compared with controls: CCL11 (eotaxin 1), IL-4, transforming growth factor (TGF)- β , SMAD7, CCL24 (eotaxin 2), TSLP, IL-10, and IFN γ (all $P > 0.05$; results not shown). Together, these data show that increased IL-33 gene expression is specifically associated with established EoE in a pediatric cohort, raising the possibility that this cytokine may contribute to disease pathology.

Serum IL-33 Concentrations Are Not Increased in Pediatric EoE

Since IL-33 expression is elevated in the esophagus of EoE patients and IL-33 has been shown to circulate in response to diverse respiratory pathology (13, 23), we measured serum levels of IL-33 in the control and EoE cohorts. Serum IL-33 concentrations ranged from 2 to 5 pg/ml and were not different between the EoE and control groups (Fig. 1B; $P > 0.05$). These data suggest that circulating IL-33 is not elevated in the chronic Th2 inflammation and pathology observed in EoE and are consistent with observations made in eosinophilic pneumonia, in which acute, but not chronic, disease is marked by increased serum IL-33 (23).

IL-33 Is Localized to Endothelial, but Not Cytokeratin- or CD45-Positive, Cells in EoE

To identify the cell type expressing IL-33 in the human esophageal mucosa, the colocalization of immunoreactive IL-33 and an epithelial marker (pan-cytokeratin), leukocyte marker (CD45), or endothelial marker [von-Willebrand factor (vWF)] was evaluated by confocal microscopy in normal and EoE esophagus (Fig. 2). IL-33 was almost undetectable in the normal esophagus (Fig. 2Aiii) but was found in nuclei of vWF-positive endothelial cells (Fig. 2F, *i* and *ii*), but not CD45- or pan-cytokeratin-negative cells in EoE (Fig. 2D and E). To evaluate this further, the disposition of IL-33-stained cells of the EoE mucosa was compared with that of eosinophils (eosin-positive) and mast cells (mast cell chymase-positive) in near-consecutive sections from EoE biopsies (Fig. 2B, *i-iii*). IL-33-positive cells (Fig. 2Cii) were distinct from both eosinophils (Fig. 2Ci) and mast cells (Fig. 2Bii).

IL-33 Is Acutely Expressed in the *A. fumigatus* Model of EoE

The *A. fumigatus* model of EoE (26) has recently been used to investigate TNF-related apoptosis-inducing ligand signaling pathways in EoE pathogenesis (10). Allergen is administered

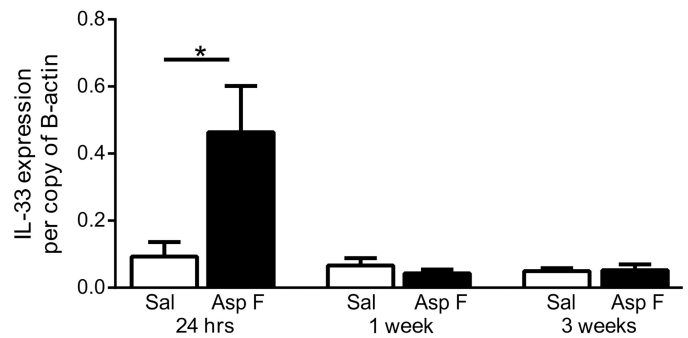


Fig. 3. IL-33 expression increases acutely, but not thereafter, in the *Aspergillus fumigatus* model of EoE. Mice were treated intranasally with saline (Sal) or *A. fumigatus* (AspF, $n = 6-8$) 3 times per week for 3 wk. Esophageal tissue was taken at 24 h, 1 wk, and 3 wk, and IL-33 expression was determined by quantitative PCR on mRNA per copy number of β -actin. Values are means $\pm$ SE. * $P < 0.05$.

intranasally thrice weekly for 3 wk, and symptoms of EoE, including eosinophilia, mast cell expansion, and esophageal epithelial and muscle cell hyperplasia, develop progressively after 1 wk, i.e., dose 6 (31). In the present study, esophageal IL-33 gene expression increased fivefold 24 h after the first *A. fumigatus* dose, returned to baseline by 1 wk, and was maintained at control levels thereafter, despite progressive development of EoE pathology (Fig. 3). The data indicate that although transient IL-33 upregulation is associated with the earliest phase of esophageal response to *A. fumigatus*, it does not accurately model the robust IL-33 induction and coincident chronic eosinophilia observed in our human cohort; therefore, an alternative model was developed.

Exogenous IL-33 Induces Transmural Inflammation and Hyperproliferation in the Mouse Esophagus

Having established that tissue IL-33 expression is increased in human EoE, we evaluated the pathological outcome in the esophagus after a cohort of mice was treated for 1 wk with recombinant IL-33 ($1 \mu\text{g} \cdot \text{mouse}^{-1} \cdot \text{day}^{-1}$ ip). Morphological evaluation of the IL-33-treated esophagus showed marked structural changes in the mucosa, including dysregulation of epithelial polarity, transmural inflammation (Fig. 4A), epithelial hyperplasia (Fig. 4B), a ~ 10 -fold expansion of the subserosal compartment area (Fig. 4B), and a modest reduction in the area of esophageal smooth muscle associated with inhibition of the muscle growth factors follistatin and insulin-like growth factor (IGF)-1 (Fig. 4, B and C). Sirius red staining of the esophageal mucosa showed no overt differences in collagen deposition by Masson's trichrome staining in any mucosal compartment after IL-33 treatment (data not shown).

Ki-67 staining in each of the epithelial, submucosal, muscularis, and subserosal compartments showed increased num-

Fig. 2. IL-33 is localized to endothelial, but not cytokeratin- or CD45-positive, cells in human EoE. Sections from controls (A) and EoE patients (B and C) were stained with hematoxylin-eosin (H&E, *i*) to confirm the presence of eosinophils in EoE biopsies (Ci, arrows) or studied by immunohistochemistry using anti-mast cell chymase (Cii) or anti-IL-33 (Ciii) on adjacent sections. To more clearly evaluate nuclear or cytoplasmic IL-33 localization, immunostaining was analyzed by confocal microscopy at high power (D-F). Nuclei of cells adjacent to blood vessels stain with IL-33 [green staining coincident with blue 4',6-diamidino-2-phenylindole (D-F)], but CD45 (red)-stained leukocytes (Di and Dii) and pan-cytokeratin (pan-CK, far-red)-stained epithelial cells (Ei and Eii) are IL-33-negative. Colocalization of IL-33 with von Willebrand factor (vWF) suggests nuclear staining of esophageal endothelial cells in EoE (Fi and Fii). epi, epithelium; bv, blood vessel.

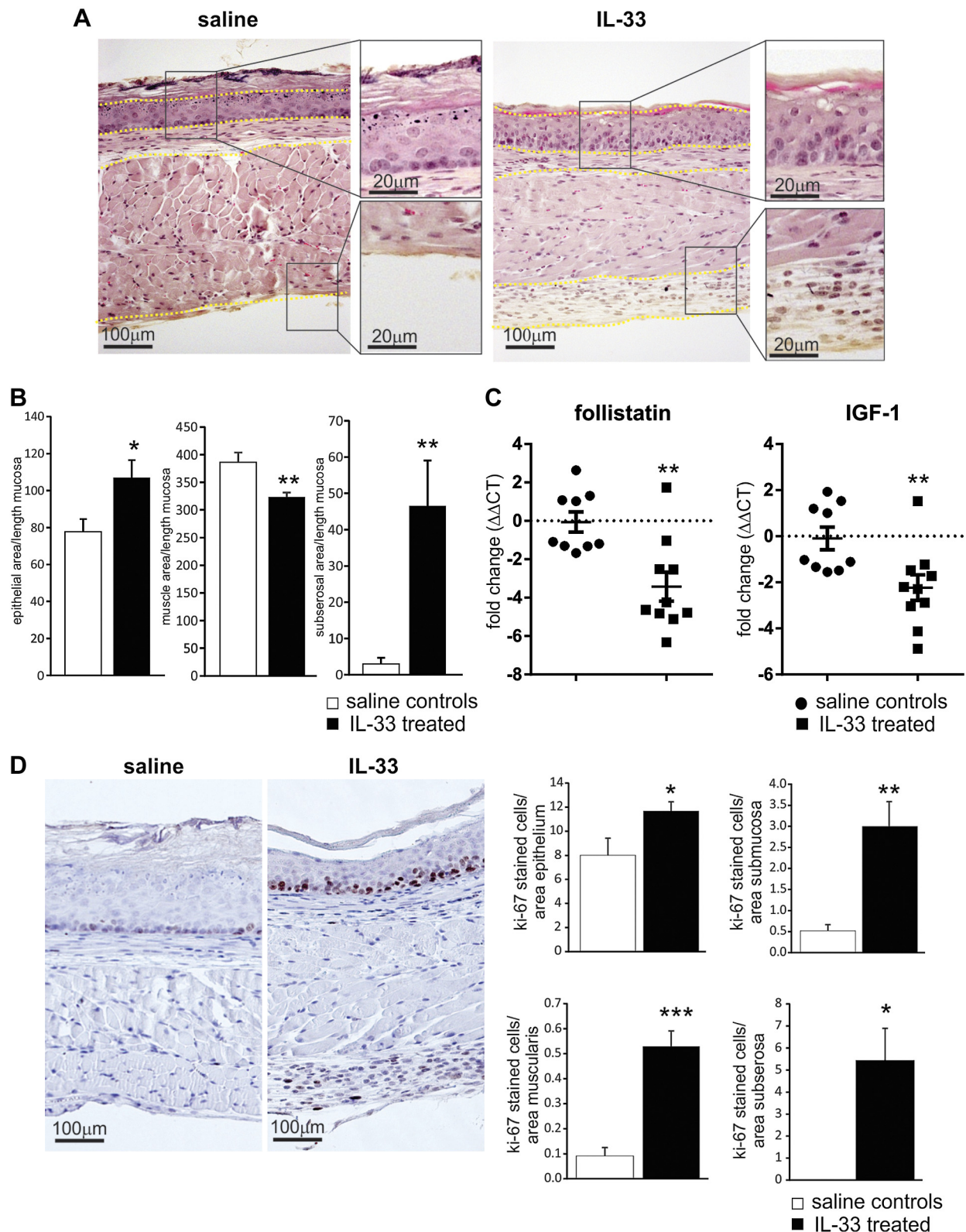


Fig. 4. IL-33 administration to wild-type mice results in transmural inflammation, epithelial hyperplasia, and muscle hypoplasia in the esophagus. Wild-type C57BL/6 mice were treated with recombinant IL-33 (1 mg day ip for 7 days, $n = 9$) or saline ($n = 9$), and tissue was subjected to morphometric or molecular analysis. **A**: discrete esophageal compartments from saline-treated control or IL-33-treated mice were quantified on hematoxylin-eosin-stained sections. **B**: epithelial, muscularis, and subserosal compartment areas. **C**: quantitative RT-PCR for follistatin and insulin-like growth factor (IGF)-1 mRNA relative to the housekeeping gene L32. Data are expressed as fold change compared with control (by the $\Delta\Delta C_t$ method). **D**: additional 4- μ m sections from saline- or IL-33-treated mice were stained with Ki-67 to assess proliferation. Density of Ki-67-stained cells was quantified in epithelium, submucosa, muscularis, and subserosa of normal (saline-treated) and IL-33-treated esophagus. Values are means $\pm$ SE. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control.

bers of stained nuclei, particularly in the epithelial and subserosal regions (Fig. 4D, *left*). Quantification of proliferation demonstrated an increase in each compartment (all $P < 0.04$; Fig. 4D, *right*). In the submucosal, muscularis, and subserosal compartments, proliferation appeared to be restricted to infiltrating immunocytes, while in the epithelial compartment, epithelial cells were hyperproliferative.

IL-33 Treatment Induces EoE and Eosinophil Chemotactic and Marker Genes

The nature of the esophageal transmural inflammation induced by IL-33 was analyzed morphologically (compare Fig. 5A, *i* and *ii*) and by quantifying expression of genes chemotactic for eosinophils, as well as eosinophil marker genes. Leukocytes in the submucosal compartment between the epi-

thelium and the muscularis were identified morphologically after hematoxylin-eosin staining and quantified per high-power ($\times 400$) field. Total leukocytes (3-fold; Fig. 5Aiii) and, particularly, eosinophils (25-fold; Fig. 5Aiv) were increased after IL-33 treatment (both $P < 0.002$). In support of these findings, the following eosinophil chemotactic or marker genes were elevated (Fig. 5B, *i-iii*): CCL11 (eotaxin 1, 3.3-fold, $P = 0.005$), CCL12 (2.5-fold, $P = 0.002$), and major basic protein (12-fold, $P = 0.001$). Expression of IL-9, IL-1 β , IL-1 α , CCL24 (eotaxin 2), IL-33 (Fig. 5Cii), and mast cell chymase (Fig. 5Ciii) was unchanged after IL-33 treatment, while TSLP expression was reduced (3-fold; Fig. 5Ci). These observations are consistent with the known activation and chemotaxis of eosinophils by IL-33 in other tissues and support a role for induction of eosinophilia in EoE by IL-33.

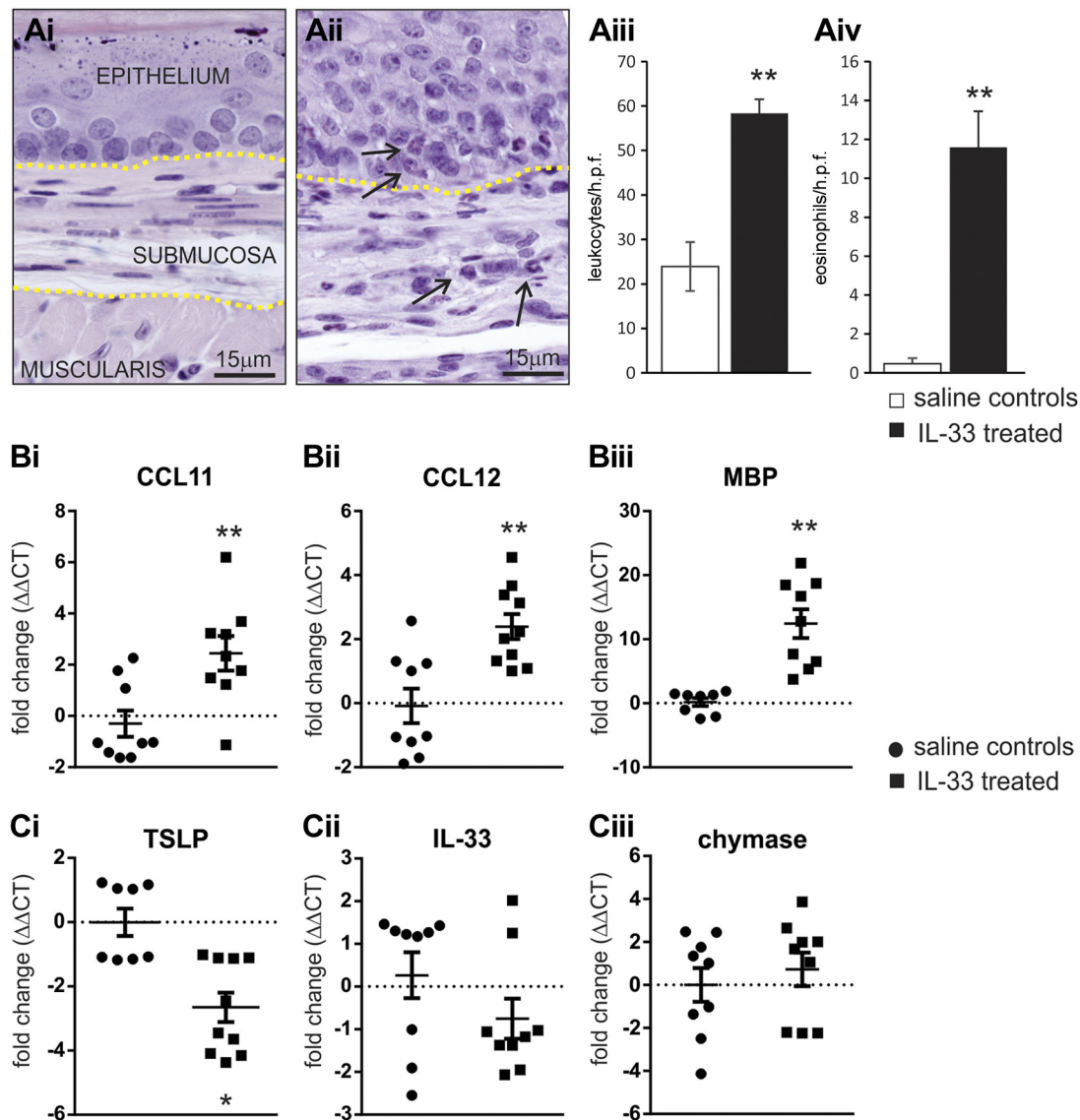


Fig. 5. Esophageal inflammation in wild-type mice treated with IL-33 is enriched in eosinophils and total leukocytes. Wild-type C57BL/6 mice were treated with recombinant IL-33 ($n = 6$) or saline ($n = 5$), and esophageal tissue was processed as described in Fig. 4 legend. Submucosa of the esophagus of saline control (Ai) and IL-33-treated (Aii) mice in this and an additional cohort (each $n = 9$ in total) was quantified for total leukocytes (Aiii) and eosinophils (Aiv). hpf, High-power field. Quantitative RT-PCR for CCL11 (Bi), CCL12 (Bii), and major basic protein (MBP, Biii) or for thymic stromal lymphopoietin (TSLP, Ci), IL-33 (Cii), and mast cell chymase (Ciii), as well as the housekeeping gene L32, was performed, and data are expressed as fold change compared with control (by the $\Delta\Delta C_t$ method). ** $P < 0.01$ vs. saline control.

IL-33 Suppresses Treg Cell Induction and Expression of Marker Genes

Unlike other gut epithelia, we found that Treg cell markers were strongly inhibited by exogenous IL-33 compared with saline controls (Fig. 6). While CD4 expression was increased along with the increase in total leukocytes (8-fold, $P = 0.02$; Fig. 6*i*), we observed reduced expression of CD25/IL-2 receptor- α (-22 -fold, $P < 0.001$; Fig. 6*ii*), Foxp3 (-250 -fold, $P = 0.001$; Fig. 6*vi*), inducible T-cell costimulator (-12 -fold, $P < 0.002$; Fig. 6*iv*), and cytotoxic T-lymphocyte-associated protein 4 (-10 -fold, $P < 0.001$; Fig. 6*v*). Treg cell differentiation and the regulatory factors TGF β (-4 -fold, $P < 0.01$; Fig. 6*viii*) and IL-10 (-2.5 -fold, $P = 0.07$; Fig. 6*vii*) were also inhibited or strongly trended to reduction, while the IL-33 receptor ST2, which is expressed by Treg cells and numerous other cell types, was unchanged ($P > 0.05$; Fig. 6*iii*). Thus global IL-33 application *in vivo* in the mouse profoundly suppresses Treg cell markers as well as the key differentiation factor TGF β , raising the possibility of a reduced capacity for mucosal tolerance in the esophagus induced by local IL-33 release.

IL-33 Treatment Induces a Th2/ILC2-Biased Cytokine Response, Enhances a Selective Th1/17 Response, and Activates M2 Macrophages in the Mouse Esophagus

IL-33 has been shown to induce a Th2/ILC2-biased transcription factor and cytokine gene signature in lung, intestine, and stomach when chronically stimulated locally or delivered exogenously (7, 21, 44). We quantified a Th2/ILC2 gene marker panel in the IL-33-treated mouse esophagus and found increased mRNA expression of GATA3 (5-fold, $P = 0.02$; Fig. 7*Ai*), IL-13 (3,800-fold, $P = 0.004$; Fig. 7*Aii*), and amphiregulin (7-fold, $P = 0.05$; Fig. 7*Aiii*); however, IL-5 expression was reduced (-3 -fold, $P = 0.004$; Fig. 7*Aiv*), and IL-4 expression was unchanged ($P > 0.3$; data not shown). Consistent with reduced Treg cell activity in response to IL-33 and, likely, a direct secondary response to IL-33, the Th1 cytokine IFN γ was increased 10-fold ($P = 0.02$; Fig. 7*Bi*), the Th17 cytokine IL-17A was increased 7-fold ($P = 0.04$; Fig. 7*Bii*), and the Th17 regulator IL-23 was increased 2-fold ($P = 0.04$; Fig. 7*Biii*). IL-17B ($P = 0.39$) and IL-17F ($P = 0.69$) were unchanged (data not shown). Finally, the alternatively activated (M2) macrophage markers Ym1 and arginase-1 were

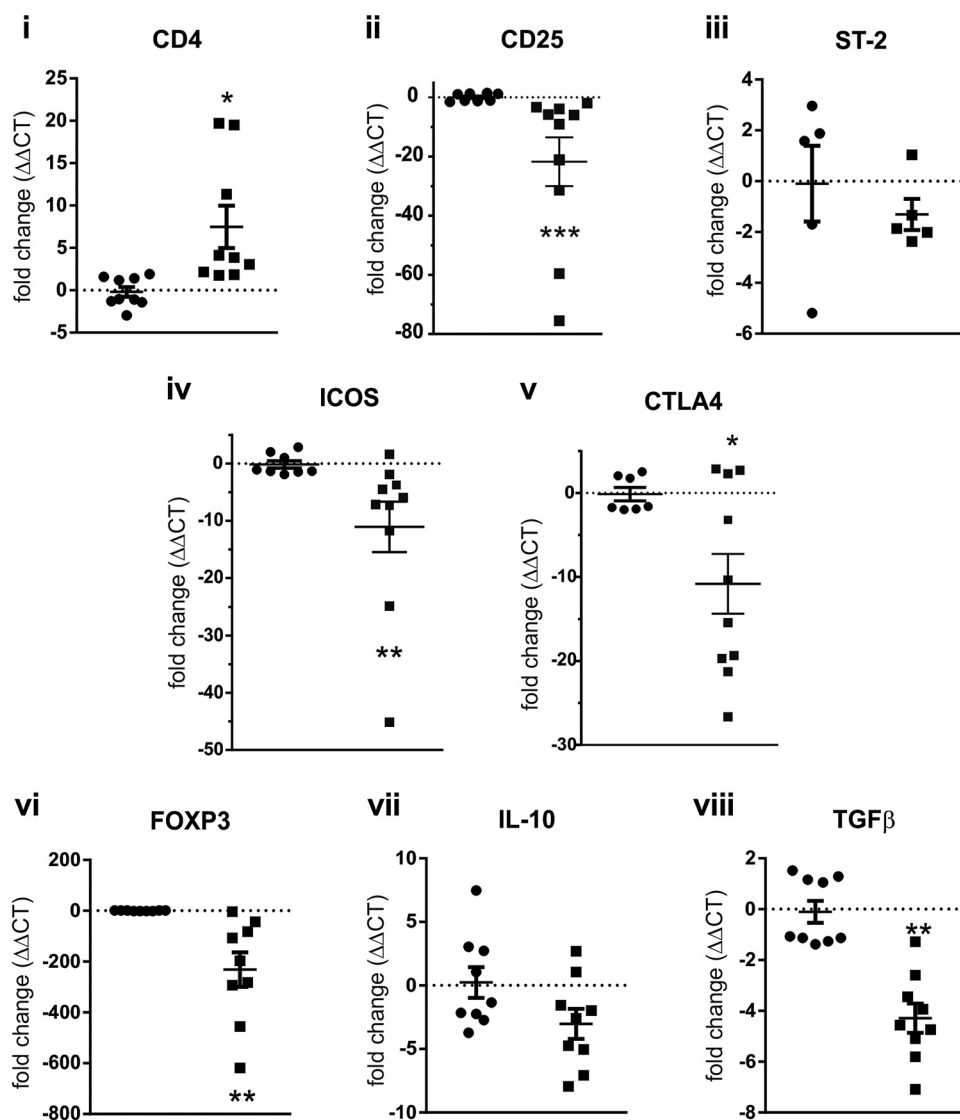


Fig. 6. IL-33 administration depletes expression of regulatory T (Treg) cell markers and differentiation factors. To assess Treg cells, quantitative RT-PCR of CD4 (*i*), CD25 (*ii*), ST2 (*iii*), inducible T-cell costimulator (ICOS, *iv*), cytotoxic T-lymphocyte-associated protein 4 (CTLA4, *v*), Foxp3 (*vi*), IL-10 (*vii*), and transforming growth factor- β (TGF β , *viii*) was performed, and data are expressed as fold change compared with control (by the $\Delta\Delta CT$ method). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. saline control.

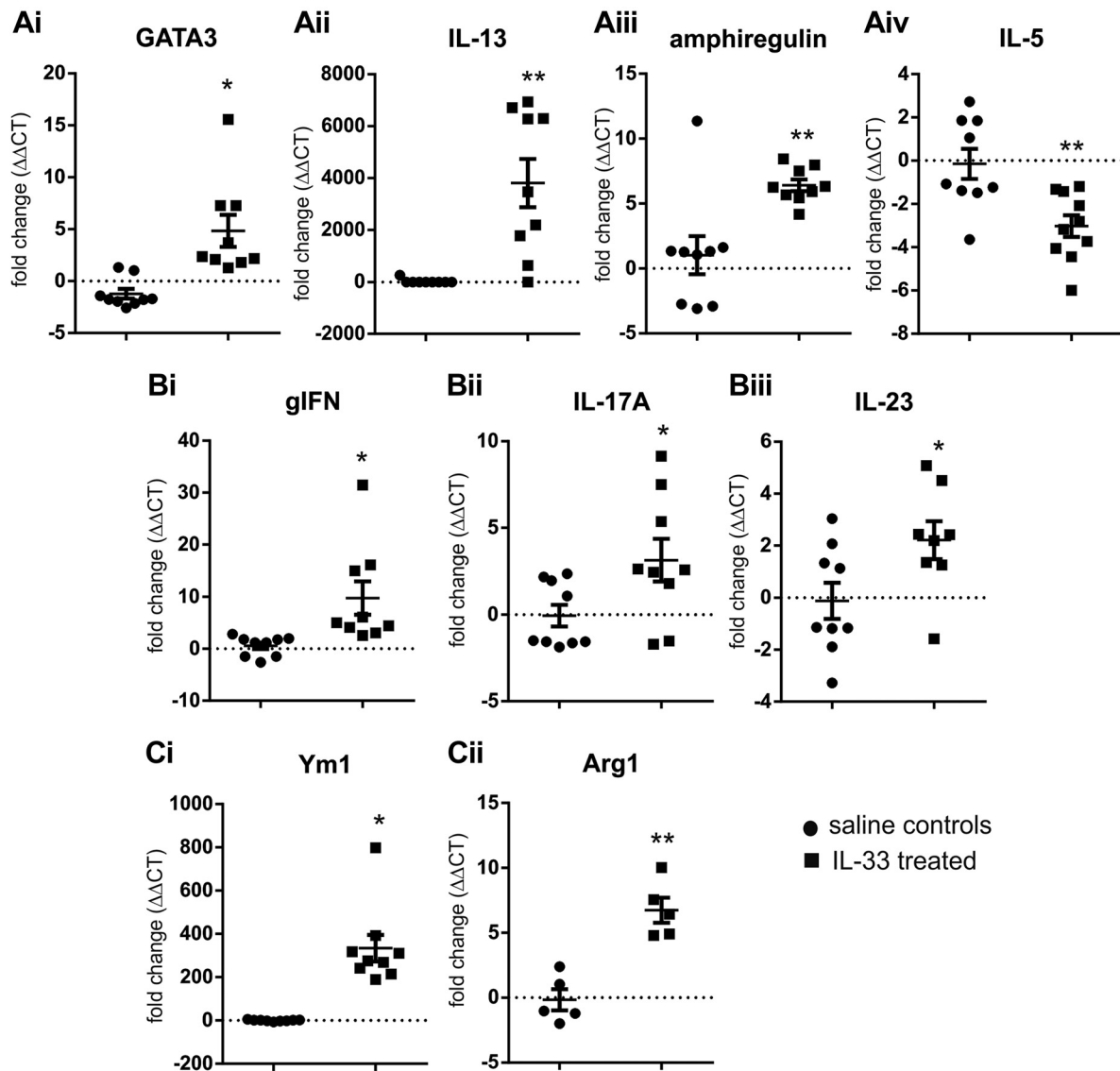


Fig. 7. Esophageal inflammation in mice treated with IL-33 shows elevation of innate lymphoid cell group 2 (ILC2), Th1/17, and M2 macrophage markers. Wild-type C57BL/6 mice were treated with recombinant IL-33 or vehicle, and esophageal tissue was processed as described in Fig. 4 legend. RNA was extracted from the esophagus of saline control and IL-33-treated mice, and quantitative RT-PCR was used to quantify the ILC2/Th2 markers GATA3 (Ai, $n = 9$), IL-13 (Aii, $n = 9$), amphiregulin (Aiii, $n = 9$), and IL-5 (Aiv, $n = 9$); the Th1/Th17 markers γ -IFN (Bi, $n = 9$), IL-17A (Bii, $n = 9$), and IL-23 (Biii, $n = 9$); and the M2 macrophage markers Ym1 (Ci, $n = 9$) and arginase-1 (Arg1, Cii, $n = 5$) relative to the housekeeping gene L32. Values (means $\pm$ SE) are expressed as fold change compared with controls (by the $\Delta\Delta C_t$ method). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control.

increased 320-fold ($P = 0.04$; Fig. 7Ci) and 5-fold ($P < 0.001$, $n = 5$; Fig. 7Cii), respectively. These data show that IL-33 treatment for 1 wk can induce a marked Th2/ILC2 gene profile in the mouse esophagus that is heavily biased toward IL-13 and away from IL-5 and, at the same time, can induce a selective proinflammatory IFN γ , IL-17A, IL-23, and M2 macrophage phenotype. Future studies are required to determine the temporal relationships between Treg cell inhibition and IFN γ induction.

IL-33-Induced Eosinophilia Is Mediated by IL-13

Since application of IL-33 produces a profound inflammation of the mouse esophageal mucosa, marked by strong eosinophilia and an IL-13-based Th2 response, we evaluated the requirement for IL-13 expression by dosing WT and

IL-13 $^{-/-}$ mice with IL-33 as described above. BALB/c mice were used for this experiment, since IL-13 $^{-/-}$ mice were not available on a C57BL/6 background. The inflammatory and, thus, pathological responses to IL-33 in BALB/c mice were at least commensurate with those in C57BL/6 mice. Histological outcomes are shown in Fig. 8A, *i–iv*, and quantification of total leukocytes and eosinophils is shown in Fig. 8B, *i* and *ii*. Neither WT nor IL-13 $^{-/-}$ mice dosed with saline showed evidence of inflammation (Fig. 8A, *i* and *ii*), with fewer than 10 leukocytes or 2 eosinophils per high-power ($\times 400$) field. As expected, IL-33 treatment of WT mice (Fig. 8Aiii) produced transmural inflammation and dysregulation of the epithelial compartment, as well as an increase in total leukocytes (compare Fig. 8Bi with Fig. 5Aiii), which was reduced by 50% in IL-13 $^{-/-}$ mutants.

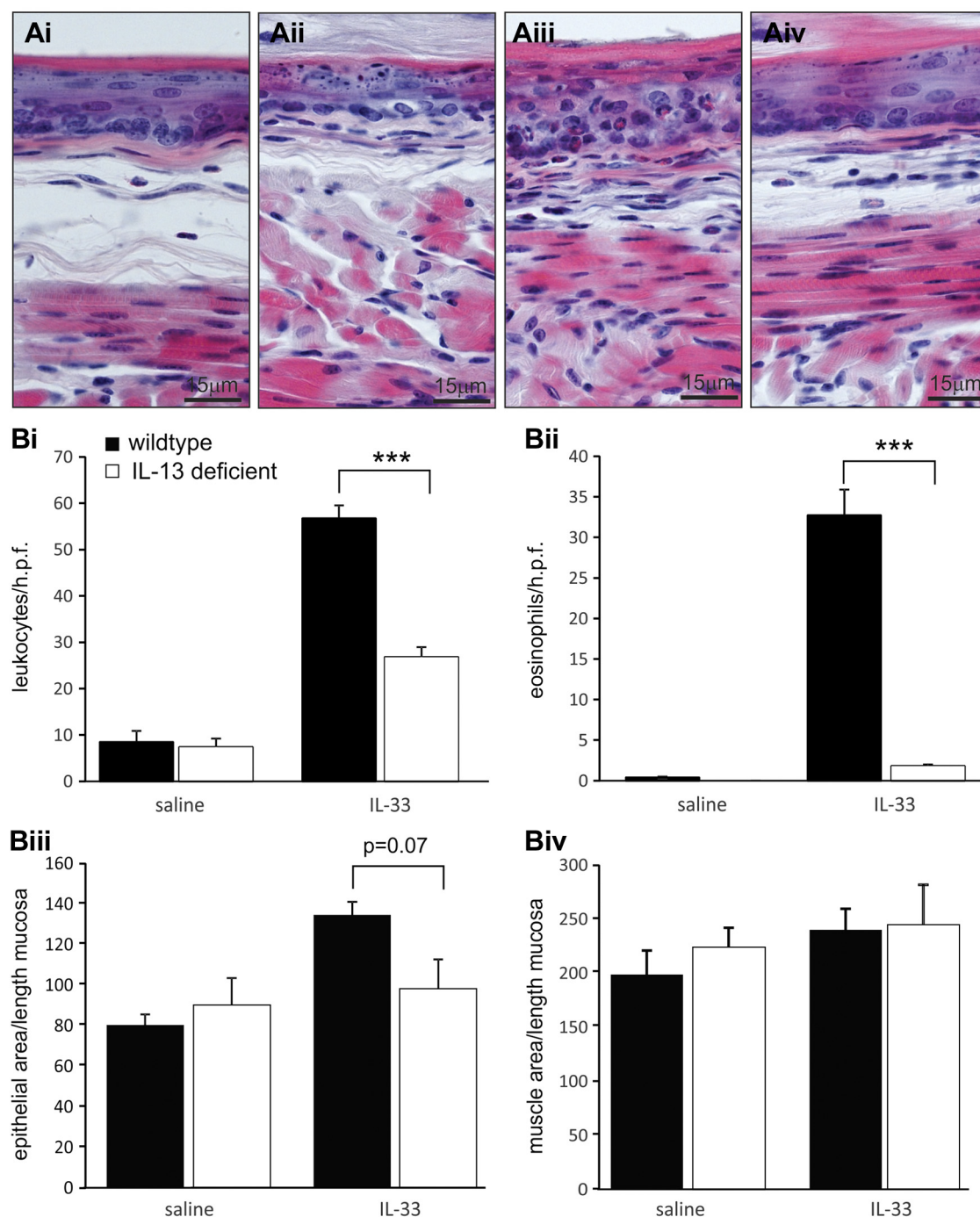


Fig. 8. IL-33-induced EoE is dependent on IL-13 expression. Wild-type and IL-13^{-/-} mice were treated with recombinant IL-33 (1 mg day ip for 7 days), and esophagi were processed for histology ($n = 5$ for all groups). Submucosa and mucosa of wild-type (Ai and Aiii) and IL-13^{-/-} (Aii and Aiv) mice treated with IL-33 (Aiii and Aiv) or saline (Ai and Aii) were assessed for total leukocytes (Bi), eosinophils (Bii), epithelial area (Biii), and muscle area (Biv). Values are means $\pm$ SE. *** $P < 0.001$ vs. control (saline).

IL-33-treated WT mice on a BALB/c background showed a strong EoE (Fig. 8Aiii), with 32 ± 4 eosinophils per high-power field, nearly three times as many as in C57BL/6 mice (compare Fig. 8Bii with Fig. 5Aiv); however, this response was strongly inhibited in IL-13^{-/-} mice. These data show that IL-33-driven EoE in BALB/c mice is mediated by IL-13; however, IL-13 only partially accounts for IL-33 induction of other leukocytes.

Short-Term Application of IL-33 Does Not Activate Mast Cells in the Mouse Esophagus

It is well established that IL-33 activates tissue mast cells, and we have shown that IL-33 induction is coincident with increased mast cell chymase staining in human EoE. Therefore, the gene expression profile of mast cell chymase was quantified in control and IL-33-treated mice (Fig. 5Ciii). Chymase mRNA

expression was unchanged by IL-33 treatment ($P = 0.94$, $n = 9$), at least over the short experimental time course employed in our study.

DISCUSSION

Here we provide evidence that the IL-1 family cytokine IL-33 contributes to the induction of an esophageal Th2 cell inflammatory response and the subsequent eosinophilia associated with EoE pathology in mice and likely also in humans (Table 3). IL-33 mRNA was elevated in a pediatric EoE cohort in association with IL-5, IL-13, and eotaxin 3 (CCL26), all key cytokines or chemokines that promote disease progression (4, 27). IL-33 is a well-established physiological regulator of IL-5 and IL-13, and its unique receptor subunit ST2 is expressed on eosinophils (9, 39), as well as other cell types, suggesting that it may promote both migration and activation of these cells directly, as well as indirectly via Th2 cell ligands.

IL-33 protein was absent from the normal pediatric esophagus but was localized immunohistochemically to the esophageal mucosa in EoE. Immunopositive cells were confined to the endothelium, being distinct from eosinophils, mast cells, epithelial cells, and leukocytes on the basis of characteristic colocalization of cell-specific markers. IL-33 staining was nuclear and colocalized with vWF in cells adjacent to blood vessels and consistent with many human tissues (29). Nuclear IL-33 staining has been linked with alarmin function (29), as also recently shown by us in the stomach (7) and by others in the intestine (35). In this study, IL-33-immunopositive cells were detected only in EoE, suggesting that it is either actively contributing to disease pathology or expressed as a by-product of EoE development. Given the well-characterized role of IL-33 in exacerbation of inflammatory disease in models of acute colitis (12), chronic inflammatory bowel disease with fibrosis (21), rheumatoid arthritis (24), and psoriasis (40), the former is more likely.

Although circulating IL-33 has been detected in atopic and eosinophilic disease, it could be measured only at low concentrations in our pediatric patients, and we were unable to detect differences in plasma IL-33 between EoE patients and controls. This is perhaps not surprising, since elevated plasma IL-33 is associated with acute eosinophilic pneumonia (13), and our entire EoE patient cohort had well-established disease. Nonetheless, plasma IL-33 may be diagnostic in the early stage of EoE induction, and so this deserves further investigation in a larger cohort, including detailed optimization of plasma handling and extraction conditions.

IL-33 application in C57BL/6 mice induced a profound eosinophilic infiltration into the esophagus, particularly adja-

cent to the serosa, in the submucosa immediately luminal to the muscularis mucosa, and also into the epithelium. This was accompanied by other pathological changes, including epithelial hyperproliferation with loss of pseudostratification and increased transmucosal infiltration of leukocytes. Coincident with this morphology, major basic protein, a gene marker of eosinophils, and chemotactic mediators, such as CCL11 and CCL12, were significantly elevated. CCL26, or eotaxin 3, is the most upregulated gene in human EoE and is diagnostic for this disease (4); however, it is a pseudogene in the mouse (33), with early eosinophilic chemotaxis mediated by CCL11 via IL-13 induction (30). Of interest in this context is the observation that IL-13 expression is highly responsive to IL-33 application: it is increased ~4,000-fold in the mouse esophagus after IL-33 treatment. IL-13 is a Th2 cytokine that is a key mediator of EoE and, alone, can recapitulate the EoE phenotype in mice after intratracheal application (3). Application of IL-33 in IL-13^{-/-} mice confirms that the early EoE induced by IL-33 is mediated by IL-13; however, this cytokine accounted for only about half of the leukocyte infiltrate, suggesting an additional direct role for IL-33 in driving noneosinophilic transmural inflammation in murine EoE.

Another significant finding of this study was that TSLP was unchanged in the pediatric EoE cohort compared with normal esophagus and that it was inhibited fourfold after IL-33 application in the mouse. These findings suggest that murine eosinophilia, and possibly eosinophilia in our pediatric cohort, was a prime regulatory effect of IL-33 independent of TSLP induction. They also suggest that TSLP drive may not be required in all cases of EoE, although, like IL-33, TSLP can induce a mucosal Th2 cell response, and polymorphisms of TSLP are associated with EoE development (34). In this regard, it will be interesting to compare the clinical presentation and IL-33 expression profile of pediatric EoE cases with and without raised TSLP in the future. It is possible that high local IL-33 concentrations may inhibit TSLP expression either directly or via an intermediary. For instance, it has recently been demonstrated that IL-17A specifically inhibits TSLP production in human skin explants (5), consistent with the substantial increase in this Th17 cytokine (but not IL-17B and IL-17F) induced by exogenous IL-33 treatment of the mouse esophagus in our study.

IL-33 is a well-established inducer of Th2 cytokines from Th2 lymphocytes (11) and ILC2 (42). ILC2 characteristically expresses the transcription factors retinoid-related orphan receptor- α , GATA3, the cytokines IL-5, IL-13, and IL-9, and numerous cell surface markers including ST2 (42). The growth factor amphiregulin is also associated with ILC2 activation (20) but may be expressed more broadly by Th2 and Treg cells (6, 46). Treatment of mice with exogenous IL-33 potentially induced GATA3, IL-13, and amphiregulin in the esophagus, but IL-5 expression was suppressed, while IL-9 and retinoid-related orphan receptor- α were unchanged. GATA3 is associated with CD4⁺ differentiation into Th2 cells (16), as well as ILC2 development and maintenance (18). Both of these lineages express IL-13, and in IL-33-treated mouse esophagus other ILC2 markers are mostly unchanged, so the relative contributions of Th2 and ILC2 to EoE development remain unclear. IL-5 is established as a key effector cytokine in experimental models of EoE (27, 28); in humans, IL-5 expression correlates with the extent of eosinophilia in EoE and

Table 3. *Characteristics of pediatric EoE and mouse esophageal phenotype induced by IL-33*

Pediatric EoE	Mouse Esophageal Phenotype Induced by IL-33
Marked eosinophilia	Marked eosinophilia
Mucosal remodeling	Mucosal remodeling
Epithelial hyperproliferation	Epithelial hyperproliferation
Eotaxin, IL-5, and IL-13 increased	Eotaxin and IL-13 increased, IL-5 reduced
TSLP expression unchanged	TSLP expression reduced
Mast cell markers elevated	Mast cell chymase unchanged

IL-33 was administered intraperitoneally daily for 1 wk. EoE, eosinophilic esophagitis.

disease activity, and we have shown here that it is upregulated in a human pediatric cohort. Application of neutralizing antibodies to IL-5 in clinical studies of EoE have produced equivocal results, which suggest that other factors, including IL-9 and its target mast cell, might contribute to EoE pathogenesis. Our data extend this hypothesis to accommodate IL-33 as a candidate promoter of EoE in at least a subset of pediatric patients, the effects of which are independent of either IL-5 or IL-9 and may be mediated mainly by IL-13.

A major and unexpected finding from our study was the profound inhibition of the expression of Treg cell activation markers and differentiation regulators in the mouse esophagus after IL-33 treatment. Characteristic Treg cell markers CD25/IL-2 receptor- α , Foxp3, inducible T-cell costimulator, cytotoxic T-lymphocyte-associated protein 4, and the regulators TGF β and IL-10 were strongly suppressed or trended strongly to suppression in IL-33-treated mice. These observations, in conjunction with increased Th1/17 activation, are consistent with a regulatory mechanism for this inhibition and a previous study showing inhibition of Foxp3⁺Treg and stimulation of T-effector cells in an allergen model of EoE in mice (47). Unfortunately, we have not been able to confirm whether IL-33 treatment produces a smaller Treg cell population (as opposed to reduced activity markers alone), since flow cytometric analysis of isolated mouse esophageal Treg cells was inconsistent due to the very small number of Treg cells per mouse. Nonetheless, it is clear that Treg cells are normally responsible for the induction of immune tolerance in autoimmune and allergic disease and, in the intestine (35), heart (43), spleen and thymus (25), and colon (11), can be induced in an IL-33/Th2-dependent fashion after dendritic cell activation and secretion of IL-2 (25). Our results and those of Zhu et al. (47) demonstrate that the opposite is true in the mouse esophagus, in which reduced IL-2 production or receptor expression correlates with inhibition of Treg cell induction. The regulatory mechanisms for these tissue-specific and opposing actions of IL-33 have yet to be clarified. However, since EoE development is thought to come about as an allergic response to trigger foods or ingested aeroallergen, a plausible mechanism for its induction, on the basis of our observations and when confirmed in human esophagus, is loss of tolerance induced by IL-33-dependent deactivation and/or depletion of Treg cells.

Application of recombinant IL-33 to various mucosae has been shown to induce muscle hypertrophy/hyperplasia, a condition also associated with advanced EoE pathology (8). In the intestine, IL-33-induced muscle growth develops in an IGF-1 and TGF β -dependent fashion (44, 15), and muscle hypertrophy can also be induced by IL-4/IL-13 induction of M2 macrophages (46). Conversely, in the esophagus we have demonstrated that application of IL-33 for 1 wk induced a modest reduction in cross-sectional muscle area, consistent with the inhibition of both follistatin and IGF-1, as well as TGF β . Whether these changes are reversed after chronic IL-33 treatment and whether the acute inhibition of muscle area is direct or via an intermediary need to be addressed in the future.

Cumulatively, our data support the view that IL-33 may have an important role in early EoE etiology and disease progression. It can stimulate eosinophil migration and activation via eotaxins and IL-13 and can activate ILC2 and M2 macrophages. In contradistinction to other mucosae and in keeping with its pleiomorphic nature, IL-33 strongly inhibited

Treg cell markers and modestly reduced muscle hypertrophy in an IGF-1/follistatin-dependent fashion. Finally, the effects of IL-33 may be direct via ST2 on target cells or indirect via production of Th2-promoting cytokines and chemokines, especially IL-13, which is an established mediator of EoE. When developed clinically, IL-33 inhibitors may prove to be important members of the therapeutic arsenal utilized to treat EoE in humans.

ACKNOWLEDGMENTS

We thank Prof. Eric Reynolds (Melbourne Dental School, University of Melbourne) for providing IL-13^{-/-} mice.

GRANTS

This study was supported by a project grant from the National Health and Medical Research Council (Australia) and the Victorian Government's Operational and Infrastructure program.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.M.J., R.G.H., and A.S.G. developed the concept and designed the research; L.M.J., R.G.H., T.R.M., J.B., N.O.-S., D.I.P., L.O., K.A.G., O.H., M.S., A.M.C., J.M., and K.J.A. performed the experiments; L.M.J., J.B., A.M.C., J.M., and A.S.G. analyzed the data; L.M.J. and A.S.G. interpreted the results of the experiments; L.M.J., A.M.C., and A.S.G. prepared the figures; L.M.J., R.G.H., and A.S.G. drafted the manuscript; L.M.J., T.R.M., and A.S.G. edited and revised the manuscript; L.M.J., R.G.H., T.R.M., J.B., N.O.-S., D.I.P., L.O., K.A.G., O.H., M.S., K.J.A., and A.S.G. approved the final version of the manuscript.

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