

Anti-IL-5 therapy reduces mast cell and IL-9 cell numbers in pediatric patients with eosinophilic esophagitis

Iris M. Otani, MD,^{a,e} Arjun A. Anilkumar, BS,^{a,e} Robert O. Newbury, MD,^{b,d} Monica Bhagat, MD,^{a,e} Lisa Y. Beppu, BS,^{a,d} Ranjan Dohil, MD,^{c,d} David H. Broide, MB, ChB,^{a,e} and Seema S. Aceves, MD, PhD^{a,d,e} San Diego, Calif

Background: Eosinophilic esophagitis (EoE) is a clinicopathologic entity of increasing worldwide prevalence. IL-5 is essential for eosinophil trafficking, and anti-IL-5 therapy decreases esophageal eosinophilia. EoE is associated with prominent mast cell infiltration.

Objective: We investigated whether anti-IL-5 (mepolizumab) treatment reduced esophageal mast cell accumulation in biopsy specimens from pediatric patients with EoE from a previous randomized anti-IL-5 trial.

Methods: A subanalysis was completed for children treated with 0.55, 2.5, or 10 mg/kg mepolizumab monthly for 12 weeks followed by no treatment until week 24. Quantitative immunohistochemistry was used to assess the numbers of eosinophils, tryptase-positive mast cells, IL-9⁺ cells, and mast cell–eosinophil couplets before and after treatment.

Results: Forty-three biopsy specimens had adequate tissue for paired analysis. Forty percent of subjects responded to anti-IL-5 (defined as <15 eosinophils per high-power field [hpf] after mepolizumab therapy), and 77% of all subjects had decreased numbers of mast cells after anti-IL-5. In responders epithelial mast cell numbers decreased from 62 to 19 per hpf ($P < .001$), were significantly lower than in nonresponders after therapy ($P < .05$), and correlated with eosinophil numbers ($r = 0.75$, $P < .0001$). Mast cells and eosinophils were found in couplets before therapy, and these were significantly decreased only in responders after anti-IL-5 ($P < .001$). Esophageal eosinophils comprised the majority of cells that made the mast cell growth factor IL-9. IL-9⁺ cell numbers decreased from 102 to 71 per hpf ($P < .001$) after anti-IL-5.

Conclusions: Pediatric patients with EoE had significantly fewer mast cells, IL-9⁺ cells, and mast cell–eosinophil couplets in the esophageal epithelium after anti-IL-5 therapy. Because

eosinophils were one source of IL-9, they might support esophageal mastocytosis. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: Eosinophilic esophagitis, pediatric, eosinophils, mast cells, IL-5, IL-9

Eosinophilic esophagitis (EoE) is a disease of increasing worldwide prevalence. Diagnostic criteria are isolated esophageal eosinophilia of 15 or more eosinophils per high-power field (hpf) on hematoxylin and eosin stain at $\times 400$ magnification (light microscopy), especially in the context of concurrent acid blockade with proton pump inhibitor therapy.¹ Symptoms vary by patient's age, but vomiting, failure to thrive, abdominal pain, and dysphagia are common.¹ Tissue remodeling is likely to be a significant contributing mechanism for strictures, dysmotility, and food impactions.²⁻⁵ Successful therapeutic interventions in patients with EoE include elimination diets, topical corticosteroids, and amino acid formulas.⁶⁻¹⁰

IL-5 is a pivotal cytokine for eosinophilopoiesis and trafficking to target organs.¹¹ Disease-modifying effects have been seen with anti-IL-5 treatment in adults with eosinophilic asthma, chronic rhinosinusitis, Churg-Strauss syndrome, and hypereosinophilic syndrome (HES).¹²⁻¹⁵ Animal models of aeroallergen-induced EoE demonstrate that IL-5 is required for eosinophilopoiesis and trafficking into the esophagus.¹¹ Recent human studies have demonstrated that anti-IL-5 (mepolizumab) can reduce eosinophil counts in the esophagus by 54% in adults with EoE.¹⁶ A randomized trial in children treated with mepolizumab demonstrated that 32% of subjects had residual esophageal eosinophilia of less than 20 eosinophils per hpf and that 8.8% had complete eosinophil resolution after therapy.¹⁷ Similarly, a randomized controlled trial of reslizumab showed an up to 67% reduction in esophageal eosinophilia after treatment.¹⁸

Both eosinophils and mast cells play a role in EoE pathogenesis. A specific mast cell gene signature is found in both adult and pediatric EoE.^{19,20} In addition, mast cell and eosinophil products, such as TGF- $\beta 1$, can increase esophageal smooth muscle contractility.⁴ However, a single adult study of EoE did not find any decreases in numbers of esophageal mast cells after anti-IL-5 therapy at doses of 750 and 1500 mg monthly.¹⁶ Although cultured mast cells derived from cord blood progenitors and bone marrow mast cells from subjects with systemic mastocytosis and HES express the IL-5 receptor, IL-5 is considered a relatively eosinophil-specific cytokine.²¹⁻²³ As such, the effects of anti-IL-5 on mast cells are likely largely indirect (eg, through a reduction in expression of mast cell growth factors from eosinophils).

In this study we used biopsy specimens from a previously completed 3-arm anti-IL-5 trial to investigate the effects of anti-IL-5 on esophageal mastocytosis in pediatric patients with EoE.¹⁷ We report that anti-IL-5 significantly reduced tryptase-positive cell numbers in the esophageal epithelium. We demonstrate that this effect was most pronounced in responders to anti-IL-5

From the Divisions of ^aAllergy and Immunology, ^bPathology, and ^cGastroenterology and the Departments of ^dPediatrics and ^eMedicine, University of California, San Diego, Rady Children's Hospital.

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Corresponding author: Seema S. Aceves, MD, PhD, Division of Allergy and Immunology, Departments of Pediatrics and Medicine, 9500 Gilman Dr, MC-0635, La Jolla, CA 92093. E-mail: saceves@ucsd.edu.

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Abbreviations used

EoE: Eosinophilic esophagitis
 HES: Hypereosinophilic syndrome
 hpf: High-power field
 LP: Lamina propria

(defined as <15 eosinophils per hpf after therapy) and that mast cell numbers increased at week 24. Mast cells and eosinophils were found in close proximity before therapy, but numbers of these couplets were significantly decreased in responders at week 12. Double immunofluorescence demonstrated that eosinophils made IL-9 in the esophagus and that IL-9-expressing cell numbers were decreased after mepolizumab treatment. As such, eosinophils might be important instigators, propagators, or both of esophageal mastocytosis in patients with EoE.

METHODS**Human subjects**

Anonymized esophageal biopsy specimens archived during a 3-active-arm multicenter clinical trial of mepolizumab in pediatric patients with EoE (MEE103219, clinicaltrials.gov number NCT00358449; Aceves, PI UCSD site) were obtained from GlaxoSmithKline (Research Triangle Park, NC).¹⁷ Esophageal biopsy specimens from 43 of the original 57 patients were studied. In this clinical trial subjects were given monthly doses of anti-IL-5 for 3 months, followed by 3 months without therapy. Endoscopy with biopsy was done at weeks 12 and 24. Only paired tissue samples were studied ($n = 43$ for weeks 0 and 12 and $n = 41$ of these 43 at week 24). Fifty-four biopsy specimens were obtained from GlaxoSmithKline. Eleven were excluded because 8 biopsy specimens did not have adequate esophageal tissue in paired specimens before and after therapy, and 3 biopsy specimens no longer met the initial clinical trial diagnostic criteria of 20 or more eosinophils per hpf. The original study and this *post hoc* analysis were approved by the Institutional Review Board of the University of California, San Diego.

Immunostaining

Archived, formalin-fixed, paraffin-blocked esophageal biopsy specimens were used for all of the studies. All subjects ($n = 43$) had 0- and 12-week biopsy specimens and 41 of these 43 had 24-week biopsy specimens with adequate tissue for analysis (3-6 evaluable hpfs). In total, 127 biopsy specimens were analyzed per stain, unless otherwise indicated. IL-9-major basic protein double immunofluorescence could be completed on a subset of 6 subjects before and after therapy because of limited adequate residual tissue. These subjects had IL-9⁺ cells and eosinophils at similar levels to those detected in single stained slides. Residual lamina propria (LP) was not present for analysis in this subset of biopsy specimens. Biopsy specimens were immunostained with the Vectastain ABC system (Vector Laboratories, Burlingame, Calif) or Alexa immunofluorescence (Invitrogen, Carlsbad, Calif), as previously described.² Esophageal biopsy specimens were incubated with primary antibodies specific for human tryptase (1:150ab2378; Abcam, Boston, Mass; 1 hour at room temperature), IL-9 (1:100sc46654; Santa Cruz Biochemicals, Santa Cruz, Calif; overnight at 4°C), or major basic protein (1:50BMK13, MA135360; Thermo Scientific, Uppsala, Sweden; overnight at 4°C) and detected with the appropriate species of biotinylated or fluorescence-conjugated secondary antibodies. All immunohistochemical slides were counterstained with hematoxylin (Fischer Scientific, Fair Lawn, NJ) before quantitation, and immunofluorescence slides were stained with 4'-6-diamidino-2-phenylindole dihydrochloride.

Histologic evaluation

Peak numbers of tryptase-positive mast cells, IL-9⁺ cells, and eosinophil-mast cell couplets, which were defined as mast cells found within 3.4 μ m or

less (one half of the cell length of an average tryptase-positive cell) of an eosinophil, were counted in 3 to 6 separate hpfs in the epithelium at $\times 400$ magnification by an observer blind to therapy. Results are expressed as the peak mean number of cells per hpf. Image capture and analysis was done with ImagePro (MediaCybernetics, Bethesda, Md). Biopsy specimens were scored with our previously reported histology scoring tool.¹⁰ Degranulation was defined as absent (0) or present (1) based on the presence of granules outside of the eosinophilic cytoplasm.

Statistics

All statistical analyses and graphing were carried out with GraphPad Prism software (GraphPad Software, San Diego, Calif). Comparisons between cell counts of any 2 groups were made with the Mann-Whitney test for unpaired variables. Pre-post analysis was done with the Wilcoxon test for paired variables. Comparisons among 3 groups was done by using 1-way ANOVA. Correlations between groups were determined by generating Spearman correlation coefficients. A 2-tailed P value of less than .05 was considered statistically significant.

RESULTS**Anti-IL-5 and esophageal eosinophils, eosinophil degranulation, and eosinophil clusters**

For this *post hoc* analysis, we reanalyzed the eosinophil numbers and eosinophil (degranulation/clusters) and epithelial characteristics. Although these features were previously reported for the original cohort,¹⁷ this reanalysis was critical for accurately analyzing the relationship between the eosinophils and mast cells in the *post hoc* study. All of the subjects in this study had paired biopsy specimens available at weeks 0 and 12, and 41 had week 24 biopsy specimens with adequate tissue. Overall, peak eosinophil numbers in the esophagus decreased in 86% of subjects from a median of 79 per hpf (mean, 92 per hpf) before anti-IL-5 therapy to 18 per hpf (mean, 30 per hpf) after therapy ($P < .0001$). For the purposes of this study, *response* was defined as less than 15 esophageal epithelial eosinophils per hpf after 3 infusions of intravenous anti-IL-5 (mepolizumab). Among the anti-IL-5 responder group (40%, $n = 17$), eosinophil numbers decreased from a median of 74 per hpf (mean, 93 per hpf; 95% CI, 62-124 per hpf) to 7 per hpf (mean, 7 per hpf; 95% CI, 5-9 per hpf; $P < .0001$). Subjects with 15 or more eosinophils per hpf after therapy (defined as "nonresponders," $n = 26$) also had reduced esophageal eosinophil numbers from 83 per hpf (mean, 91 per hpf; 95% CI, 73-109 per hpf) to 31 per hpf (mean, 44 per hpf; 95% CI, 32-56 per hpf; $P < .0001$; Fig 1, A). The residual numbers of eosinophils at week 12 were significantly lower in the responder than in the nonresponder group ($P < .0001$; Fig 1, A). Eosinophil numbers were decreased in children in every dosing group at 12 weeks and increased at week 24 (Fig 1). LP eosinophil numbers did not decrease significantly in either responder (pretherapy mean, 16 [95% CI, -5 to 38]; posttherapy mean, 7 [95% CI, -2 to 16]; $n = 6$; $P = .5$) or nonresponder groups (pretherapy mean, 10 [95% CI, 4-15]; posttherapy mean, 9, [95% CI, 4-15]; $n = 21$; $P = .5$), but because of the small numbers of responders with adequate LP for evaluation, this should be interpreted cautiously.

The eosinophil degranulation score (0, absent; 1, present) decreased from a median of 1 (mean, 1) to 0 (mean, 0.28; $P < .0001$) only in anti-IL-5 responders. The presence of eosinophil clusters was improved in both the responder and nonresponder groups (mean score, 0.44 and 0.58 before therapy and 0 and 0.27 after therapy in responders and nonresponders, respectively). Both eosinophil features were significantly lower after

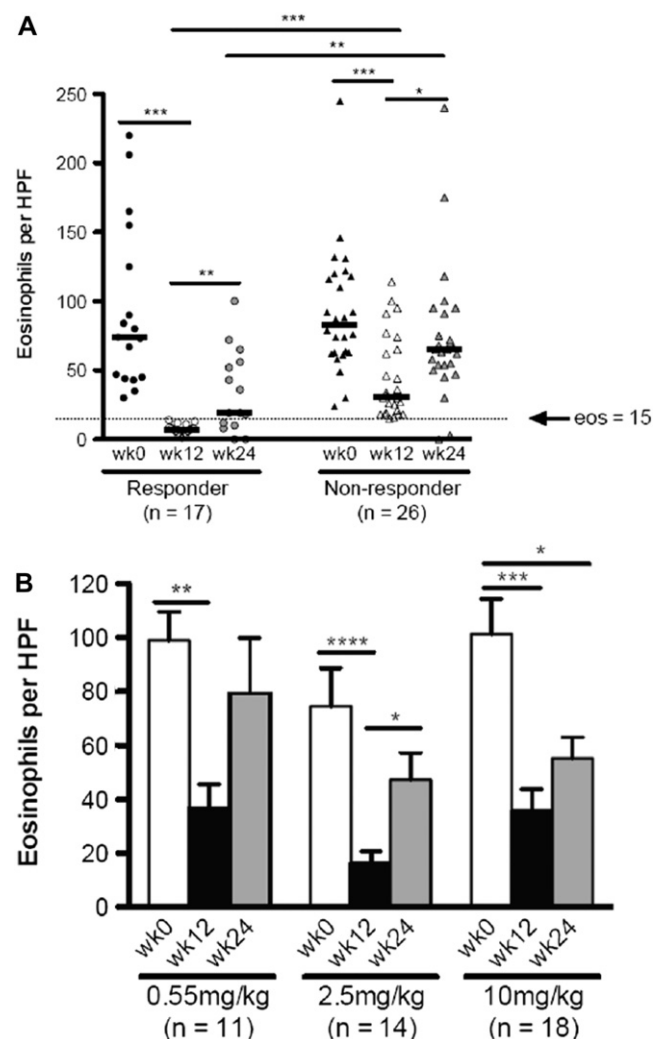


FIG 1. Anti-IL-5 decreases esophageal eosinophil numbers. Peak eosinophil numbers before and after therapy at weeks 0, 12, and 24 by response category (**A**) and dose (**B**) are shown. Responders are defined as having less than 15 eosinophils per hpf at week 12. Bars represent median eosinophil counts (Fig 1, A) and means with SEs (Fig 1, B). Fig 1, A: * $P < .01$, ** $P < .001$, and *** $P < .0001$. Fig 1, B: * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$.

therapy in responders than in nonresponders ($P < .0001$ and $P < .05$ for degranulation and clusters, respectively).

Anti-IL-5 and esophageal epithelial tryptase-positive and LP mast cells

In the overall group mast cell numbers were decreased in 33 (77%) of 43 of the patients (Fig 2, A). Among the responders (n = 17), mast cell numbers decreased from a median of 62 per hpf (mean, 64 per hpf; 95% CI, 49-79 per hpf) to 19 per hpf (mean, 25 per hpf; 95% CI, 16-35 per hpf) after therapy ($P = .0002$; Fig 2, B). By comparison, LP mast cell numbers remained static in both responders (median, 7 and 10 per hpf before and after therapy, respectively) and nonresponders (median, 7 and 9 per hpf before and after therapy, respectively; data not shown). Mast cell numbers decreased after anti-IL-5 therapy in the responder group and the 2.5 mg/kg group (Fig 2).

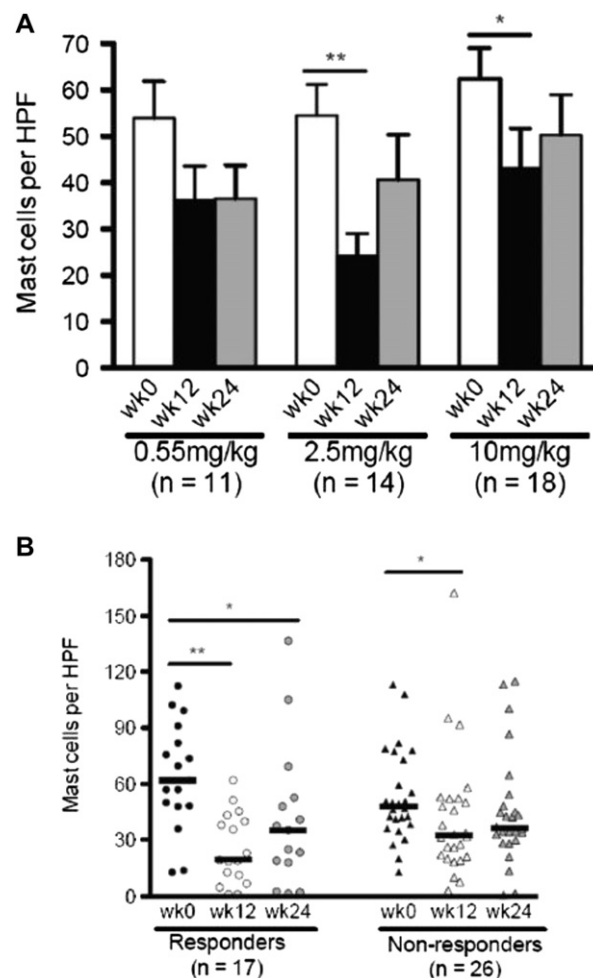


FIG 2. Anti-IL-5 decreases esophageal mast cell numbers. Peak mast cell numbers by dose (**A**) and response (**B**) at weeks 0, 12, and 24 are shown. Responders are defined as having less than 15 eosinophils per hpf at week 12. Bars represent means with SEs (Fig 2, A) and medians (Fig 2, B). Fig 2, A: * $P < .05$ and ** $P < .001$. Fig 2, B: * $P < .05$ and ** $P < .001$.

Anti-IL-5 and mast cell-eosinophil couplets

The presence of mast cell-eosinophil couplets has been demonstrated in patients with allergic rhinitis, and mast cell-eosinophil direct contact is a likely mechanism by which mutual pro-survival factors are released.²² We used double immunofluorescence to assess the presence of and quantitate the numbers of eosinophil-mast cell couplets (Fig 3, A). We defined eosinophil-mast cell couplets as a mast cell found within a distance of 3.4 μ m from an eosinophil. We analyzed all biopsy specimens with adequate tissue at 0, 12, and 24 weeks of therapy for mast cell-eosinophil couplets. Responders (n = 16) had a mean of 6.4 and 1.0 couplets per hpf before and after therapy, respectively, whereas nonresponders had a mean of 4 and 2 couplets per hpf before and after therapy, respectively ($P < .001$ for responders only; Fig 3, B). At 24 weeks, the nonresponders had 3.7 couplets, whereas the responders had 1 couplet per hpf (Fig 3, B). The numbers of couplets in the LP were low and did not change significantly in the small numbers of responders or nonresponders with adequate LP for analysis (n = 12, data not shown). Couplet numbers increased in the epithelium at week 24 in the nonresponders only (Fig 3, B). Consistent with the concept that mast

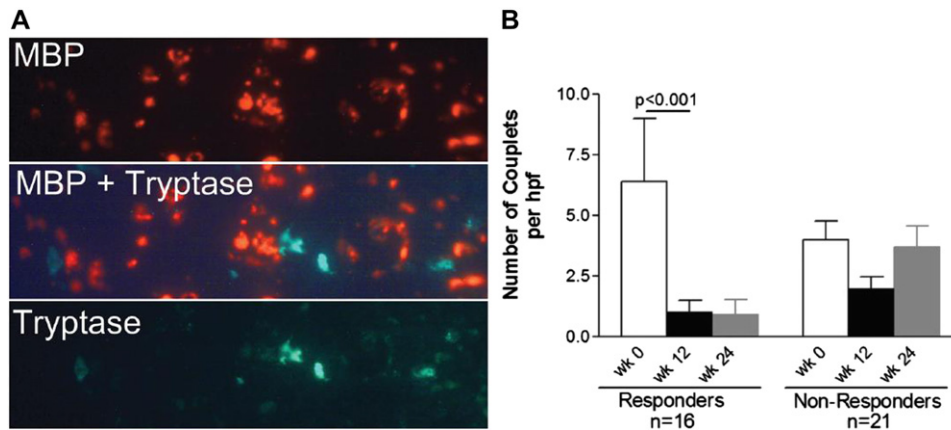


FIG 3. Anti-IL-5 decreases eosinophil-mast cell couplet numbers. **A**, Representative image showing that eosinophils and mast cells are found in close proximity in the esophagi of patients with EoE (red, major basic protein [MBP]; green, tryptase). **B**, Numbers of eosinophil-mast cell couplets before and after anti-IL-5 therapy by response at weeks 0, 12, and 24. Bars represent means with SEs.

cells and eosinophils can be in proximity and important for mutual survival/activation, eosinophil numbers correlated with mast cell numbers in the esophagi of pediatric patients with EoE who responded to anti-IL-5 treatment ($r = 0.75$, $P < .0001$, see Fig E1 in this article's Online Repository at www.jacionline.org). Re-evaluating the correlation between eosinophils and mast cells when not counting eosinophils within an eosinophil cluster demonstrated similar results ($r = 0.74$, $P < .0001$). By evaluating mast cell counts at varying eosinophil numbers at week 12, it was clear that there was a close relationship between mast cell and eosinophil numbers (see Fig E1).

Anti-IL-5 and epithelial IL-9⁺ cells

We were interested in determining whether there was a potential indirect, eosinophil-dependent mechanism by which mast cell numbers would be decreased in pediatric patients with EoE after anti-IL-5 therapy. IL-9 is a well-described mast cell growth and survival factor expressed by eosinophils and other cell types. In the overall group the number of IL-9⁺ cells decreased from a median of 107 (baseline) to 80 (week 12) per hpf ($P < .01$). The change in IL-9⁺ cell numbers was not dose dependent. There was a significant decrease in IL-9⁺ cells per hpf in the epithelium of responder patients after anti-IL-5 therapy (before therapy: median, 102; mean, 103, 95% CI, 91-115; after therapy: median, 71; mean, 66; 95% CI, 50-82; $P = .0005$; Fig 4, A-C). In addition, there was a significant difference between week 12 IL-9⁺ cell numbers in responders compared with nonresponders after therapy ($P = .02$; Fig 4, A and C). Individually, all but 2 responders had decreased IL-9⁺ cell counts per hpf after therapy (Fig 4, B). The numbers of IL-9⁺ cells increased at week 24 to 93 per hpf in the responders (Fig 4, B).

IL-9⁺ cell numbers correlated positively with mast cell numbers in responders ($r = 0.44$, $P < .01$). Double immunofluorescence demonstrated that eosinophils were one but not the only cellular source of IL-9 in patients with EoE (Fig 4, D). Eosinophil numbers correlated positively but not strongly with IL-9⁺ cells ($r = 0.34$, $P < .01$). By using the limited subset of biopsy specimens with adequate residual tissue ($n = 12$ biopsy specimens, $n = 6$ subjects), double immunofluorescence demonstrated that $70\% \pm 6\%$ of

IL-9⁺ cells were eosinophils. There was a significant decrease in epithelial IL-9⁺ eosinophil numbers and a nonsignificant trend toward reduction in noneosinophil IL-9⁺ cell numbers after anti-IL-5 therapy (see Fig E2 in this article's Online Repository at www.jacionline.org). Tryptase-positive cells were not a source of IL-9 (Fig 4, E). As such, it appears that eosinophils might be the majority but not the only cells that produce IL-9 in patients with EoE.

Relationship to clinical features

We assessed whether mast cell numbers in the esophagus associated with the symptom complex seen in patients with EoE. Reported symptoms included stomach pain, chest/throat pain, regurgitation, vomiting, sensation of food stuck in the throat, difficulty drinking, pain with drinking, difficulty eating solid food, and pain when eating solid food. These symptoms were graded on a severity scale from 1 to 6 in the original clinical trial. Subjects who demonstrated decreases in stomach pain, chest/throat pain, pain with drinking and eating solids, and/or sensation of food stuck in the throat all had statistically significant decreases in epithelial mast cell and eosinophil numbers. In the subgroup of patients with a greater than 70% decrease in mast cell numbers, numbers of mast cell correlated with the severity of stomach pain ($r = 0.56$, $P < .05$), chest/throat pain ($r = 0.51$, $P < .05$), and drinking pain ($r = 0.60$, $P < .05$). By contrast, there was no correlation between eosinophil numbers and symptom severity. No subjects were symptom free at week 12. As such, it is possible that in a subset of subjects the degree of mastocytosis correlates with a subset of clinical symptoms.

DISCUSSION

In this study we have demonstrated a number of novel findings in pediatric patients with EoE treated with anti-IL-5. Because IL-5 is considered essentially an eosinophil-specific interleukin, anti-IL-5 therapy offers a unique opportunity to assess the effects of eosinophils on disease pathogenesis in human subjects. To this end, we analyzed biopsy specimens obtained during an international, randomized, blind, multicenter pediatric 3 dose trial of mepolizumab¹⁷ and found, surprisingly, that pediatric patients

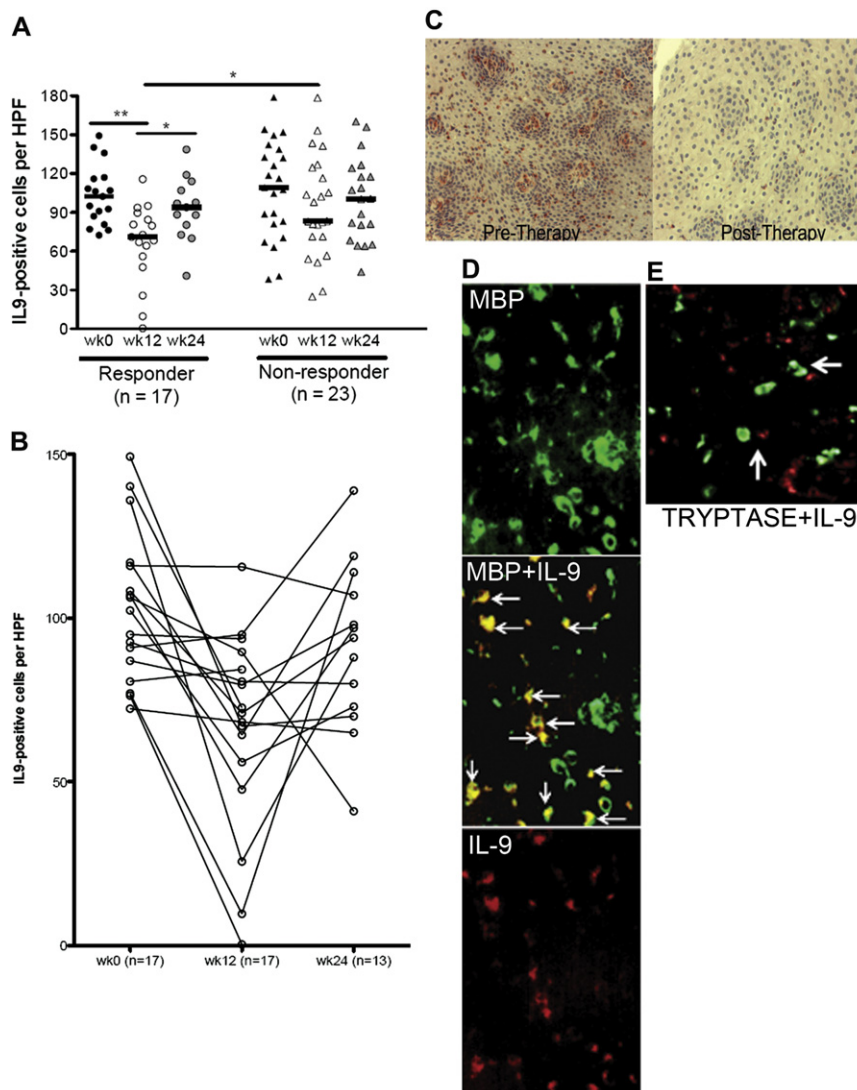


FIG 4. Anti-IL-5 decreases IL-9⁺ cell numbers. **A**, Peak IL-9⁺ cell numbers by response at weeks 0, 12, and 24. Bars represent median numbers of IL-9⁺ cells. * $P < .05$ and ** $P < .001$. **B**, Individual IL-9 counts among responders. **C** and **D**, Representative images of IL-9⁺ cells before and after anti-IL-5 (week 12) in a responder (Fig 4, C) and of major basic protein (MBP; eosinophils, green), IL-9⁺ cells (red), and overlapping cells (yellow, arrows; Fig 4, D). **E**, Mast cells (tryptase, green) do not make IL-9 (red). Arrows show mast cells and IL-9⁺ cells in proximity.

with EoE had decreased numbers of esophageal, epithelial, tryptase-positive mast cells after anti-IL-5 therapy.

This result was unexpected for a number of reasons. First, adult subjects treated with mepolizumab do not have changes in mast cell numbers after therapy. Second, mast cells in target tissues have not been shown to be directly dependent on IL-5, suggesting that the effects of anti-IL-5 would occur indirectly through a cell type that is directly affected by anti-IL-5 therapy. We also show, for the first time, that both eosinophil and noneosinophil inflammatory cells express IL-9 in the esophagi of pediatric patients with EoE and that IL-9⁺ cell numbers decrease after anti-IL-5 treatment. Lastly, we demonstrate the presence of eosinophil-mast cell couplets in biopsy specimens from pediatric patients with EoE that decrease after anti-IL-5 therapy. Together, these data suggest that esophageal eosinophils in patients with EoE might be an important component in the initiation, maintenance, or both of esophageal mastocytosis. The differences between our

pediatric observation of decreased mast cell numbers and that reported in adult subjects treated with mepolizumab could be explained by the lower doses used in the children (doses of 750-1500 mg monthly used in adults, which would correspond to dose of 10-20 mg/kg in an average adult), a difference in the anti-inflammatory responses in children who have a shorter disease duration than adults, and/or the numbers of subjects analyzed (43 children as opposed to 5 adults receiving mepolizumab).

Because decreases in eosinophil numbers correlated directly with decreases in mast cell numbers, because esophageal eosinophils are one cell type that produces IL-9, and because eosinophil-mast cell couplets are present only before therapy, eosinophils might be important to recruit, activate, and/or enhance the survival of esophageal mast cells. However, the decrease in mast cell and IL-9⁺ cell numbers was incomplete after treatment, even among responders. Our data demonstrate that both eosinophilic and noneosinophilic inflammatory cells

are IL-9 producers in patients with EoE. The majority of IL-9⁺ cells appear to be eosinophils, and as such, the decrease in IL-9⁺ cells might be due to (in large part) the direct reduction in eosinophil numbers. In contrast, mast cells do not appear to produce IL-9 in patients with EoE. In addition, it is likely that other mast cell chemoattractants and growth factors contribute to the genesis and maintenance of esophageal mastocytosis in patients with EoE. We were unable to evaluate the expression of other mast cell growth factors, such as IL-3 and GM-CSF, because of technical difficulties with the antibodies on the archived specimens. However, an analysis of stem cell factor expression demonstrated no consistent changes in staining patterns after therapy (data not shown).

It has been previously noted that mast cells and eosinophils are found in couplets in the nasal tissue of patients with allergic rhinitis.²² In addition, mast cells and eosinophils can have physical interactions allowing cross-stimulation, with eosinophil products activating mast cells and mast cell-derived mediators activating eosinophils.²² Our data show that mast cells and eosinophils tend to cluster together in the esophageal epithelium of pediatric patients with chronic EoE. Interestingly, the pattern of a single mast cell surrounded by multiple eosinophils was common and reminiscent of the complex monolayers formed in cocultures of eosinophils and mast cells.²² Quantitation of these couplets showed that there were fewer epithelial couplets in responders but not in nonresponders at week 12 of anti-IL-5 therapy. In contrast, there were no decreases in eosinophil, mast cell, or couplet numbers in the LP. These data suggest that eosinophil products could play a role in prolonging tryptase-positive, mucosal mast cell survival in patients with EoE. A subanalysis with varying mast cell cutoffs revealed that numbers could correlate with symptoms of pain. These data suggest that in some subjects the severity of mast cell inflammation might correlate with a subset of symptoms.

Cultured mast cells derived from cord blood progenitors and bone marrow-derived mast cells from patients with systemic mastocytosis and HES express IL-5 receptor.^{21,23,24} In addition, basophils express tryptase and the IL-5 receptor, and there are reports of increased activated basophil populations in the circulation of children with EoE.²⁵ As such, anti-IL-5 could also exert potential direct effects on mast cells and basophils. In addition, the effect of IL-9 on IL-5 production in mast cells has not been studied in patients with EoE. It has been shown in murine asthma models that IL-9 deficiency significantly decreases levels of mast cell progenitors in the lungs independently of IL-5²⁶ and that IL-9 induces IL-5 receptor expression on cultured eosinophils.²⁷ Thus it is possible that treatment with anti-IL-5, by decreasing IL-9-producing eosinophil numbers, decreases mast cell survival and that this might have subsequent feedback. These subjects merit further study.

In conclusion, our study suggests that eosinophils have an important role in EoE pathogenesis and are not likely to be innocent bystanders or a disease epiphenomenon in patients with EoE. Pathogenic effects of eosinophils in patients with EoE can be multipronged and include the promotion of basal cell proliferation, as well as mast cell recruitment and survival. In turn, mast cells can activate eosinophils, have damaging effects on the esophageal epithelium through the production of serine proteases, and alter smooth muscle contraction. The potentially complex interplay between mast cells, eosinophils, and IL-9 warrants further investigation in patients with EoE.

Clinical implications: 12 weeks of anti-IL-5 therapy decreases mast cell numbers in patients with EoE, which might occur through a mechanism that involves a reduction in numbers of IL-9-producing cells, including eosinophils.

REFERENCES

1. Liacouras CA, Furuta GT, Hirano I, Atkins D, Attwood SE, Bonis PA, et al. Eosinophilic esophagitis: updated consensus recommendations for children and adults. *J Allergy Clin Immunol* 2011;128:3-20.
2. Aceves SS, Newbury RO, Dohil R, Bastian JF, Broide DH. Esophageal remodeling in pediatric eosinophilic esophagitis. *J Allergy Clin Immunol* 2007;119:206-12.
3. Mishra A, Wang M, Pemmaraju VR, Collins MH, Fulkerson PC, Abonia JP, et al. Esophageal remodeling develops as a consequence of tissue specific IL-5-induced eosinophilia. *Gastroenterology* 2008;134:204-14.
4. Aceves SS, Chen D, Newbury RO, Dohil R, Bastian JF, Broide DH. Mast cells infiltrate the esophageal smooth muscle in patients with eosinophilic esophagitis, express TGF-beta1, and increase esophageal smooth muscle contraction. *J Allergy Clin Immunol* 2010;126:1198-204.
5. Kagalwalla AF, Akhtar N, Woodruff SA, Rea BA, Masterson JC, Mukkada V, et al. Eosinophilic esophagitis: epithelial mesenchymal transition contributes to esophageal remodeling and reverses with treatment. *J Allergy Clinical Immunol* 2012;129:1387-1396.e7.
6. Kagalwalla AF, Sentongo TA, Ritz S, Hess T, Nelson SP, Emerick KM, et al. Effect of six-food elimination diet on clinical and histologic outcomes in eosinophilic esophagitis. *Clin Gastroenterol Hepatol* 2006;4:1097-102.
7. Gonsalves N, Yang GY, Doerfler, Ritz S, Ditto AM, Hirano I. Elimination diet effectively treats eosinophilic esophagitis in adults; food reintroduction identifies causative factors. *Gastroenterology* 2012;142:1451-1459.e1, e14-15.
8. Liacouras CA, Spergel JM, Ruchelli E, Verma R, Mascarenhas M, Semeao E, et al. Eosinophilic esophagitis: a 10-year experience in 381 children. *Clin Gastroenterol Hepatol* 2005;3:1198-206.
9. Konikoff MR, Noel RJ, Blanchard C, Kirby C, Jameson SC, Buckmeier BK, et al. A randomized, double-blind, placebo-controlled trial of fluticasone propionate for pediatric eosinophilic esophagitis. *Gastroenterology* 2006;131:1381-91.
10. Dohil R, Newbury R, Fox L, Bastian J, Aceves S. Oral viscous budesonide is effective in children with eosinophilic esophagitis in a randomized, placebo-controlled trial. *Gastroenterology* 2010;139:418-29.
11. Mishra A, Hogan SP, Brandt EB, Rothenberg ME. IL-5 promotes eosinophil trafficking to the esophagus. *J Immunol* 2002;168:2464-9.
12. Pizzichini MM, Kjarsgaard M, Inman MD, Efthimiadis A, Pizzichini E, Hargreave FE, et al. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *N Engl J Med* 2009;360:985-93.
13. Rothenberg ME, Klion AD, Roufosse FE, Kahn JE, Weller PF, Simon HU, et al. Treatment of patients with the hypereosinophilic syndrome with mepolizumab. Mepolizumab HES Study Group. *N Engl J Med* 2008;358:1215-28.
14. Kim S, Marigowda G, Oren E, Israel E, Wechsler ME. Mepolizumab as a steroid-sparing treatment option in patients with Churg-Strauss syndrome. *J Allergy Clin Immunol* 2010;125:1336-43.
15. Gevaert P, Lang-Loidolt D, Lackner A, Stammberger H, Staudinger H, Van Zele T, et al. Nasal IL-5 levels determine the response to anti-IL-5 treatment in patients with nasal polyps. *J Allergy Clin Immunol* 2006;118:1133-41.
16. Straumann A, Conus S, Grzonka P, Kita H, Kephart G, Bussmann C, et al. Anti-interleukin-5 antibody treatment (mepolizumab) in active eosinophilic oesophagitis: a randomised, placebo-controlled, double-blind trial. *Gut* 2010;59:21-30.
17. Assa'ad AH, Gupta SK, Collin MH, Thomson M, Heath AT, Smith DA, et al. Treatment of pediatric eosinophilic esophagitis with mepolizumab: a randomized, double blind, controlled clinical trial. *Gastroenterology* 2011;141:1593-604.
18. Spergel JM, Rothenberg ME, Collins MH, Furuta GT, Markowitz JE, Fuchs G, et al. Reslizumab in children and adolescents with eosinophilic esophagitis: results of a double-blind, randomized, placebo-controlled trial. *J Allergy Clin Immunol* 2012;129:456-63.
19. Abonia JP, Blanchard C, Butz BB, Rainey HF, Collins MH, Stringer K, et al. Involvement of mast cells in eosinophilic esophagitis. *J Allergy Clin Immunol* 2010;126:140-9.
20. Hsu Blatman KS, Gonsalves N, Hirano I, Bryce PJ. Expression of mast cell-associated genes is upregulated in adult eosinophilic esophagitis and responds to steroid or dietary therapy. *J Allergy Clin Immunol* 2011;127:1307-8.
21. Dahl C, Hoffman HJ, Saito H, Schlotz PO. Human mast cells express receptors for IL-3, IL-5, and GM-CSF: a partial map of receptors on human mast cells cultured in vitro. *Allergy* 2004;59:1087-96.

22. Elishmereni M, Alenius HT, Bradding P, Mizrahi S, Shikotra A, Minai-Fleminger Y, et al. Physical interactions between mast cells and eosinophils: a novel mechanism enhancing eosinophil survival in vitro. *Allergy* 2011;66:376-85.
23. Wilson TM, Maric I, Shukla J, Brown M, Santos C, Simakova O, et al. IL-5 receptor α levels in patients with marked eosinophilia or mastocytosis. *J Allergy Clin Immunol* 2011;128:1086-92.
24. Ochi H, DeJesus NH, Hsieh FH, Austen KF, Boyce JA. IL-4 and -5 prime human mast cells for different profiles of IgE-dependent cytokine production. *Proc Natl Acad Sci U S A* 2000;97:10509-13.
25. Siracusa MC, Saenz SA, Hill DA, Kim BS, Headley MB, Doering TA, et al. TSLP promotes interleukin-3-independent basophil hematopoiesis and type 2 inflammation. *Nature* 2011;477:229-33.
26. Jones TG, Hallgren J, Humbles A, Burwell T, Finkelman FD, Alcaide P, et al. Antigen-induced increases in pulmonary mast cell progenitor numbers depend on IL-9 and CD1d-restricted NKT cells. *J Immunol* 2009;183:5251-60.
27. Gounni AS, Gregory B, Nutku E, Aris F, Latifa K, Minshall E, et al. Interleukin-9 enhances interleukin-5 receptor expression, differentiation, and survival of human eosinophils. *Blood* 2009;96:2163-71.

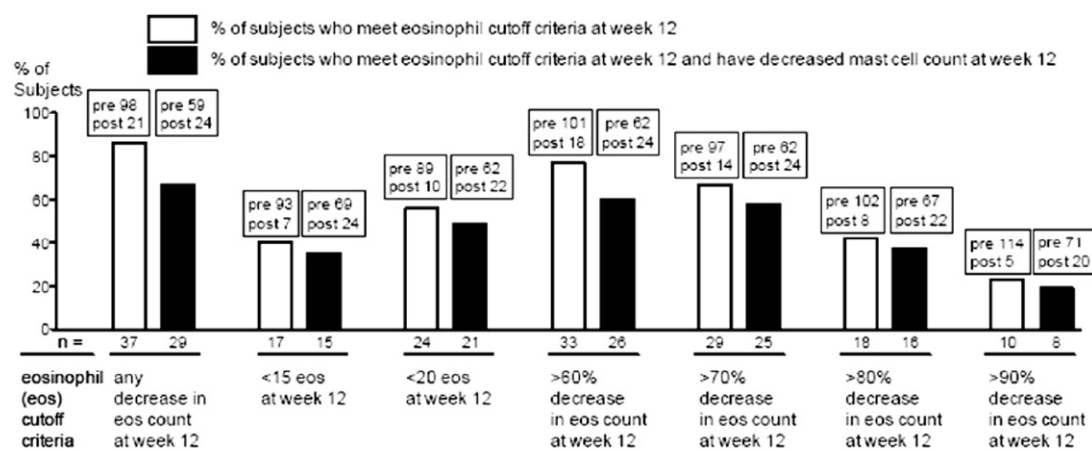


FIG E1. Eosinophil and mast cells numbers have a close relationship. The percentage of subjects with varying decreases in numbers of esophageal epithelial eosinophils (*open bars*) and mast cells (*solid bars*) in the given eosinophil category. The mean peak numbers of eosinophils and mast cells before (*pre*) and after (*post*) anti-IL-5 are indicated above the respective bars.

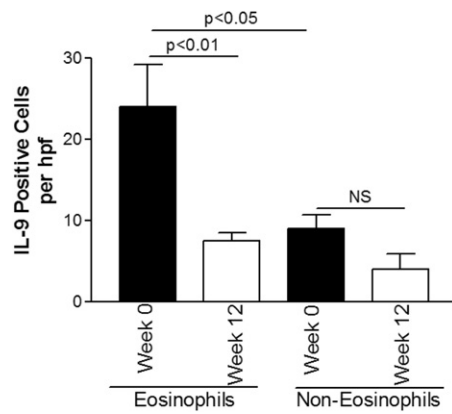


FIG E2. There is a significant decrease in IL-9-producing eosinophil numbers and a nonsignificant decrease in noneosinophilic cell numbers after anti-IL-5 therapy (n = 6 subjects before and after mepolizumab).