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A striking local esophageal cytokine expression profile in eosinophilic esophagitis¹

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Abstract

Background—Eosinophilic esophagitis (EE) is an emerging worldwide disease that mimics gastroesophageal reflux disease.

Objective—Early studies have suggested that esophageal eosinophilia occurs in association with T helper type 2 allergic responses, yet the local and systemic expression of relevant cytokines has not been well characterized.

Methods—A human inflammatory cytokine and receptor PCR array containing 84 genes followed by PCR validation and multiplex arrays were used to quantify cytokine mRNA in esophageal biopsies and blood levels.

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Clinical implication: The pathogenesis of EE involves a dysregulated local cytokine network in the esophageal mucosa and elevated eotaxin-3 expression (89% sensitivity in a single biopsy), in the absence of consistent systemic changes in cytokines.

Results—Esophageal transcripts of numerous chemokines [e.g. *CCL1*, *CCL23*, *CCL26* (eotaxin-3), *CXCL1*, and *CXCL2*], cytokines (e.g. *IL13* and *ABCF1*), and cytokine receptors (e.g. *IL5RA*) were induced at least 4-fold in individuals with EE. Analysis of esophageal biopsies (n=288) revealed that eotaxin-3 mRNA level alone had 89% sensitivity for distinguishing EE from non-EE individuals. The presence of allergy was associated with significantly increased esophageal expression of *IL4* and *IL5* mRNA in active EE patients. We identified 8 cytokines (*IL-4*, *IL-13*, *IL-5*, *IL-6*, *IL-12p70*, *CD40L*, *IL-1 α* , and *IL-17*) whose blood levels retrospectively distinguished 12 non-EE from 13 EE patients with 100% specificity and 100% sensitivity. When applied to a blinded, prospectively recruited group of 36 patients, the cytokine panel scoring system had a 79% positive predictive value, 68% negative predictive value, 61% sensitivity, and 83% specificity for identifying EE.

Conclusion—Evidence is presented that *IL13* and *IL5* associate with eosinophil and eotaxin-3 levels, indicating the key role of adaptive Th2 immunity in regulating eotaxin-3-driven esophageal eosinophilia in the absence of a consistent systemic change in cytokines.

Keywords

Eosinophilic Esophagitis; Eosinophils; Cytokines; Biomarkers; Eotaxin-3/CCL26; Allergy

Introduction

Eosinophilic esophagitis (EE) is an emerging worldwide disease characterized by marked esophageal eosinophil infiltration (≥ 15 eosinophils/hpf) that is not responsive to acid suppressive therapy.(1–3) Symptoms mimic gastroesophageal reflux disease, vary with age, and include vomiting, abdominal pain, dysphagia, and food impactions.(1;4) While several phenotypic subsets of EE patients have emerged, EE esophageal transcriptome analysis has revealed a highly conserved expression profile irrespective of patient phenotype (as defined by gender, atopic status, and familial clustering) although the sensitivity of the EE transcriptome has not been determined.(5;6) The diagnosis of EE (also abbreviated EoE in some publications), requires endoscopy with biopsy analysis since reliable, non-invasive biomarkers have not yet been identified. Blood levels of eosinophils, eotaxin-3, EDN, and IL-5 proteins are elevated in EE, but their sensitivity and specificity are too low to be clinically helpful.(7)

Early studies in mice have established that esophageal eosinophilia occurs in Th2 inflammatory responses,(8–10) but the expression of Th2 cytokines in EE patients is reported in only a few studies.(11–18) Characterization of gene expression differences between EE and normal (NL) patients via esophageal microarray expression analysis established eotaxin-3 as the most over-expressed gene in EE patients; this finding has been replicated in independent studies.(19–21) Immunological cytokines are often produced at levels below the detection of genome-wide expression chips. For example, *IL13* is not part of the initial EE transcriptome identified by Affymetrix DNA chip analysis of esophageal tissue;(14) however, we have identified a 16-fold increase in esophageal *IL13* of EE patients compared with control individuals using real-time PCR.(15) In this study, we examined the expression of a panel of potentially relevant cytokines in esophageal biopsies from a cohort of EE and NL patients and then replicated select genes in an independent large cohort. Furthermore, we investigated the relationship between these cytokines, particularly their correlation with eotaxin-3 and *IL5*, two cytokines already established to be essential in EE pathogenesis.(22) We also sought to determine how clinical parameters (atopy, allergic status, and eosinophil levels) affected the expression of these genes. Finally, we quantified plasma cytokine levels and tested their relevance in the diagnosis of EE compared to unaffected allergic and non-allergic controls.

Methods

Sample collection and patient characteristics

In this study, 3 cohorts were studied for RNA expression levels: 1) The discovery cohort was composed of a selection of 5 NL and 5 untreated EE patients, 2) The replication cohort was composed of 11 NL and 11 EE patients selected for absence of any steroid treatment, 3) Finally, we examined a large cohort composed of 288 biopsies collected over 3 years [EE, n = 226; NL, n = 14; GERD or CE (chronic esophagitis), n = 14 with a mean = 6.4, median = 4.5, range [1–16] eosinophils/hpf; missing or other diagnosis, n = 34 were not included in the study] and classified EE patients based on the number of eosinophils/hpf (in at least 1 hpf), when available, into active (≥ 24 eosinophils/hpf, n = 97), intermediate (1–23 eosinophils/hpf, n = 49) or inactive (0 eosinophils, n = 52) EE; patients with steroid treatment and/or dietary management were included in these groups. Patients diagnosed with gastroesophageal reflux disease or non-EE chronic esophagitis were regrouped in the CE group. A proportion (47%) of our 226 EE patients was treated with proton pump inhibitor (PPI) at the time of the endoscopy. Out of the patients who did not receive PPI treatment at the time of the endoscopy (n = 120), the patients either did not respond to a treatment including PPI (13%) or the patients did. respond to steroids alone (11%), diet management alone (39%) or the combination of the steroids and diet management (33%) in a future endoscopy. No information was available for 5 patients. Additionally, plasma from the blood of non-EE (including NL, CE, and GERD) and EE patients was used to quantify cytokines in three cohorts: 1) a learning set (n = 25) composed of 12 NL and 13 EE patients, 2) a before-and-after treatment set (n = 5) composed of EE patients, and 3) a prospectively recruited blinded set of patients referred for endoscopy composed of non-EE and active EE patients, excluding treated and partially treated EE patients (n = 36). For research purposes, active EE was defined as ≥ 24 eosinophils/hpf in at least 1 hpf. Blood samples were collected in heparinized tubes and centrifuged (3000 rpm) for 10 min at 4 °C; plasma was stored at –70 °C until further use. The allergic status was defined as having present or past history of allergic diseases and/or at least 1 positive skin prick testing. Biopsy and blood samples were collected during routine endoscopy or blood draw after informed consent as approved by the Institutional Review Board.

RNA extraction and RT-PCR analysis

Total RNA from biopsy samples were stored in RNALater before being extracted using the mini RNA extraction Kit (Qiagen), and reverse transcription was performed using Iscript (Bio-Rad). The reactions for each set of samples were done at different times and produced a different yield, leading to variations in the detection limits of the different sets. Real-time PCR was performed by rapid-cycling using the ready-to-use IQ5 SYBR mix (Bio-Rad) according to the manufacturer's instructions. The primer set sequences and PCR conditions are available upon request. PCR products were sequenced using the CCHMC sequencing core facility.

The Human Inflammatory Cytokine and Receptor Array PAHS-011 (SABiosciences) was employed in 5 NL and 5 EE patients. The complete list of the 84 genes of PAHS-011 is available in supplementary methods.

Multiplex analysis for cytokine quantification

For the learning set and the before-and-after treatment set, samples were run in duplicate in the 29-plex Lincoplex human cytokine from Millipore. For the prospectively recruited blinded set, patients were collected prospectively and the investigator was unblinded only at the end of the analysis. These samples were subjected to the 39-plex Milliplex human

cytokine panel. The complete list of cytokines quantified in these cohorts, detection limits, and reproducibility are available in supplementary methods.

A scoring system based on a panel of cytokines was established, adding 1 to a patient's score for each upregulation or downregulation of specific cytokines: 1) upregulation, having a value higher than the maximum value (pg/mL) observed in the NL (IL-1 α > 753, IL-4 > 967, IL-5 > 7, IL-6 > 155, IL-13 > 281) and 2) downregulation, having a value below the minimum value (pg/mL) observed in NL (CD40L < 2986) or below the average observed in NL when at least 1 NL was below the detection limit (IL-12p70 < 24, IL-17 < 15) (Supplementary Table I). Any patients reaching a score of 3 or more were classified as EE with 100% sensitivity and 100% specificity (Supplementary Table II).

Statistical analysis

Data are expressed as mean $\pm$ SD. Statistical significance comparing 2 different treatments or groups was determined by the Student's *t* test (normal distribution, equal variance), Welch's *t* test (normal distribution, unequal variances), Mann-Whitney test (nonparametric test, two groups), Kruskal-Wallis test followed by a Dunn's multiple comparison test (nonparametric test, three groups or more) or paired *t* test (for quantification of cytokines before and after therapy in the same patients) using Prism 4 Software. Nonparametric (ranked) correlations were calculated using Spearman correlations. Linear regressions were then calculated and *p* values assessed to test the hypothesis that a linear correlation exists with a slope different from 0.

Results

Cytokine and cytokine receptor mRNA expression in NL and EE patients

Using the Human Inflammatory Cytokine & Receptor PCR Array, the expression of 84 key genes involved in the inflammatory response were quantified in esophageal biopsies from a discovery cohort with 5 representative EE and 5 representative NL control patients (Table 1). Out of the 84 genes present on the array, 21 gene expressions were modified by more than 4-fold (19 upregulated and 2 downregulated) in EE compared to NL patient biopsies, and 1 gene was significantly downregulated but not modified by more than 4-fold (Table 1). The upregulated genes included eotaxin-3 (69-fold); ATP-binding cassette, sub-family F, member 1 (18-fold); chemokine (C-X-C motif) ligand 1 (GRO- α) (16-fold); chemokine (C-C motif) ligand 23 (MIP3) and *IL1B* (7-fold each); *IL1F9* (6-fold); *CD40L*, *CXCL2*, *CCR5*, and *CXCL3* (5-fold each); and *IL5RA*, *CCL1*, *CCL20*, *BCL6*, and *IL17C* (4-fold each). The down-regulated genes were chemokine (C-X-C motif) ligand 14 (BRAX) (9-fold); IL-1 family, member 6 (epsilon) (4-fold); and IL-1 receptor antagonist (*IL1RN*) (2-fold). Interestingly, eotaxin-3, *IL8*, *CXCL1*, and *IL1B* were found to be upregulated in our prior studies using microarray analysis; nevertheless, in this study we identified dysregulation of several other genes that were not previously suspected (Table I). Although increased by more than 4-fold, few genes reached significance, likely due to the sample size (NL, *n* = 5; EE, *n* = 5). Most gene expression levels were confirmed by real-time PCR and reached significance in a replication cohort with a larger sample size (NL, *n* = 11; EE, *n* = 11). In the replication cohort, the differential expression of most of the genes identified in the discovery cohort was substantiated, including *IL1B*, *IL1RN*, *IL5RA*, and *CCL1* (Table 1 and Figure 1).

Cytokine expression as a function of the activity of the disease

We were next interested in testing whether the mRNA level of the most upregulated cytokine (*IL13*), chemokines (e.g. eotaxin-3), and receptor (*IL5RA* and its ligand *IL5*) would vary with the degree of activity and within patient groups. Using real-time PCR on a large

cohort of patients ($n = 288$), eotaxin-3 mRNA was the most robust gene overexpressed in active EE patients [9.7×10^{-3} (3.3×10^{-3} - 1.7×10^{-2}) median (25–75 interquartile), Supplementary Table III] compared to NL controls [3.7×10^{-4} (6.1×10^{-5} - 4.6×10^{-4}) $p < 0.005$]. In this population, only 5 EE patients had an eotaxin-3 expression level that overlapped with NL levels, indicating 89% sensitivity. The activity of the disease was an important factor since partially treated (intermediate) EE patients, with an intermediate level of eosinophils (1–23 eosinophils/hpf) [2.8×10^{-4} (7.2×10^{-5} - 1.0×10^{-3})], and successfully treated (inactive) EE patients, with no esophageal eosinophils (0 eosinophil/hpf) [1.1×10^{-4} (4.8×10^{-5} - 4.1×10^{-4})], did not have significant increases in the eotaxin-3 levels compared to the NL group. *IL13* was significantly upregulated in active EE compared to NL [6.7×10^{-4} (2.5×10^{-4} - 2.2×10^{-3}) versus 8.3×10^{-5} (4.0×10^{-5} - 1.2×10^{-4}) with 19.5% overlap]. Similarly to eotaxin-3, *IL13* levels in intermediate [1.1×10^{-4} (4.3×10^{-5} - 2.2×10^{-4})] and inactive EE [1.6×10^{-5} (1.0×10^{-5} - 8.2×10^{-5})] were not significantly different from NL levels (Figure 2). The *IL5RA* mRNA expression level was significantly upregulated in active EE compared to NL patients or to inactive EE patients. Notably, *IL5RA* mRNA expression was not detectable in 64% of NL patients tested, 50% of inactive EE, 33% of intermediate EE, and 22% of active EE. The expression of its ligand, *IL5*, followed the same trend: *IL5* mRNA was significantly increased in active EE patients versus NL patients and was lower in intermediate and inactive EE patients compared to active EE patients.

As a control, *IL4* mRNA showed no significant differences in active EE compared to NL overall; however, *IL4* mRNA levels were significantly decreased by therapies (glucocorticoids or allergen removal). Additionally, *IL2* mRNA was not modified in active EE compared to NL patients (data not shown). No significant correlation between eotaxin-3 expression and eosinophil number was observed in the partially treated EE patient group. Only 4 intermediate EE patients, with 5–15 eosinophils/hpf, had eotaxin-3 expression levels that reached the lower interquartile of eotaxin-3 expression in active EE patients. No significant difference was observed between the NL and CE groups for *IL13*, eotaxin-3, *IL5RA*, *IL5*, and *IL-4* (Figure 2), and sensitivity to distinguish CE from EE was similar to that of NL from EE (89%).

***IL4* and *IL5* esophageal mRNA level as a function of the allergy status**

We hypothesized that Th2 cytokine levels in active EE patients (> 24 eosinophil/hpf) would correlate with a past or present history of allergy, potentially with higher levels in atopic individuals. Indeed, *IL4* and *IL5* had significantly increased mRNA levels in allergic EE patients compared to non-allergic EE patients [2.2×10^{-5} (6.0×10^{-6} - 9.8×10^{-5}) versus 4.8×10^{-6} (3.6×10^{-6} - 5.9×10^{-6}) $p < 0.0005$ and 1.2×10^{-4} (2.9×10^{-5} - 3.1×10^{-4}) versus 3.3×10^{-5} [8.3×10^{-6} - 8.7×10^{-5}] $p < 0.005$, respectively]. No significant change in levels of eotaxin-3 or *IL13* (Figure 3) was found in allergic versus non-allergic EE patients [1.3×10^{-2} (8.3×10^{-3} - 2.3×10^{-2}) versus 5.8×10^{-3} (3.0×10^{-3} - 1.4×10^{-2}) and 6.7×10^{-4} (2.5×10^{-4} - 2.1×10^{-3}) versus 5.1×10^{-4} (2.4×10^{-4} - 2.1×10^{-3}), respectively] (Figure 3). No significant change in *IL2* mRNA was observed as a function of the allergy history (data not shown). These results suggest that *IL4* and *IL5* are dysregulated in allergic EE patients compared to non-allergic EE patients and reflect the systemic allergic history of the patients rather than the activity of the disease.

Correlation of cytokine expression in active EE patients

We were interested in testing the hypothesis that abnormal cytokine levels would correlate with each other in patients with active EE. In particular, we predicted that *IL13* would likely correlate with other Th2 cytokines as well as eotaxin-3 since IL-13 has been shown to induce the latter cytokine. Indeed, we found a significant Spearman correlation between *IL13* and eotaxin-3 (Spearman $r = 0.55$, $p = 0.0002$) and between *IL5* and eotaxin-3 (0.55 , p

= 0.0001) and a surprisingly high correlation between *IL13* and *IL5* ($r = 0.72$, $p < 0.0001$) (Figure 4). A weak correlation was found between the expression of *IL13* and *IL4* ($r = 0.32$, $p < 0.05$), and no significant correlation was found between *IL4* and eotaxin-3 ($r = 0.18$, $p > 0.05$) or between *IL4* and *IL5* ($r = 0.09$, $p > 0.05$). Linear regression analysis was significant when comparing *IL13* with either eotaxin-3 or *IL5*. No significant linear regressions were seen between *IL4* and *IL13*, *IL5* and eotaxin-3, *IL4* and eotaxin-3 or *IL4* and *IL5*. Interestingly, *IL5RA* mRNA levels were not correlated with any of the cytokine mRNA quantified and did not correlate with eosinophil number in the active EE group ($r = -0.0229$, $p = 0.85$, Supplementary Table IV). Taken together, these results suggest that *IL13* mRNA expression is highly correlated to *IL5* and eotaxin-3 expression.

Blood cytokines levels in EE patients

We were interested in exploring the possibility that systemic levels of cytokines were abnormal in EE. Cytokine levels of non-EE (NL, $n = 12$) and active EE patients (EE, $n = 13$) (Supplementary Table I, Figure 5, Supplementary Figure 1) were quantified using a human cytokine panel multiplex assay containing 84 cytokines. Interestingly, IL-13, IL-4, IL-6, IL-5, CD40L, IL-12p70, and EGF were significantly modified in EE compared with NL (Figure 5A) and allowed discrimination of the patient diagnosis with 100% sensitivity and specificity. Cytokines were also quantified in 5 patients in active and inactive stages of the disease, and no difference in the average or paired analysis was observed between active and inactive EE even for cytokines significantly different in NL versus EE (Figure 5B). In this learning set of patients, cytokine levels were significantly decreased for IL-10, IL-1R α , and VEGF (data not shown). These results suggest that the activity of the disease does not consistently affect these systemic cytokine levels. We used the scoring panel designed for our learning set (Supplementary Table II) to predict diagnosis of prospectively recruited patients. Blinded blood plasma samples from 36 patients underwent analysis (Supplementary Table V). The scoring system identified 14 potential EE patients out of the 36 tested; 22 samples were predicted to belong to non-EE patients (Table II). After the diagnosis was revealed and linked with the data, 3 out of the 14 positive patients were non-EE patients, indicating a 79% positive predictive value. Out of the 22 patients suspected to be normal, 15 were truly negative, indicating a 68% negative predictive value. Indeed, specificity of the test was 83% with 3 false-positives out of the 18 non-EE patients tested. In conjunction, 7 out of the 18 EE patients were not identified by the test, demonstrating 61% sensitivity (Table III). The test was also able to diagnose past or present history of allergy among all the patients, regardless of the esophageal diagnosis, with a 78% positive predictive value, 32% negative predictive value, 70% specificity, and 42% sensitivity. Finally, no significant differences were observed for most cytokines between NL and EE patients. Collectively, these results suggest that EE is not characterized by a reproducible, consistent dysregulation of blood cytokine levels.

Discussion

In this study, we investigated cytokine expression levels in non-EE and EE patients in the esophageal mucosa and the blood. We identified that new cytokines not previously associated with EE, such as *IL1F9* and *CCL23*, were upregulated in EE compared to NL patients. While patchiness is an issue in EE diagnosis, we found that only 8.7% of active EE samples had an eotaxin-3 level that overlapped with NL samples using only one biopsy sample per patient. We found that mRNA levels of the Th2 cytokines *IL13*, *IL5*, and eotaxin-3 correlated with each other but that *IL4* did not correlate with *IL13* or eotaxin-3 levels. Notably, the allergic status was an important confounder as *IL4* and *IL5* mRNA were increased in allergic EE patients. Except for the eosinophil level, none of the clinical parameters analyzed (therapy, allergic status, gender) was able to explain inter-patient

variability of eotaxin-3 and IL-13 levels in active EE patients. The establishment of a scoring panel based on plasma levels, including 8 cytokines, was able to predict diagnosis with 79% positive predictive value, 68% negative predictive value, 83% specificity, and 61% sensitivity in this population of patients referred for endoscopy.

Herein, we analyzed cytokine levels of nearly 300 patients and assessed the overlap among cytokine levels. To our knowledge, this is the first time that such a large EE cohort has been studied for molecular parameters. Using real-time PCR, we demonstrated with 89% sensitivity that eotaxin-3 mRNA expression in EE patients is increased compared to control patients. Previous histopathological studies indicate that a minimum of 5 biopsies are required to achieve 100% sensitivity for diagnosis of EE with one biopsy only achieving 55% sensitivity;(23;24) our results were obtained using only one RNA sample per patient, suggesting that molecular diagnosis is a relatively promising and sensitive method for disease diagnosis.

Cytokine correlations reveal the concerted expression of *IL13*, *IL5*, and *IL4* mRNA and suggest expression in the same cell type, such as a Th2 cell producing IL-13 and IL-5. We have recently shown that IL-13 specifically induces eotaxin-3 in esophageal epithelial cells, (15) and a recent study by Prussin et al. has emphasized the presence of unique food antigen-specific, IL-5-positive Th2 cells in patients with eosinophil-associated gastrointestinal disorders (EGID) compared with patients with food anaphylaxis.(11) The implications of IL-5 and IL-13 in EE have also been demonstrated in murine EE models.(8–10) While *IL5RA* mRNA was upregulated in active EE patients, its low expression level may explain why it did not correlate with eosinophil levels. Finally, *IL4* and *IL5* are dysregulated in allergic EE patients compared to non-allergic EE patients, and these increases may reflect the systemic allergic history of the patients rather than the local activity of the disease as reflected by eotaxin-3 and IL-13 expression levels.

Yamazaki et al. have shown that common food and environmental allergens induce increased production of IL-13 and IL-5 by peripheral blood mononuclear cells after stimulation with aeroallergens or food allergens in EE patients compared to healthy individuals.(25) In the present study of patients referred for endoscopy, the establishment of a plasma scoring panel composed of 8 cytokines was able to predict diagnosis of EE with 79% positive predictive value, 68% negative predictive value, 83% specificity, and 61% sensitivity. While evidencing relatively high scores, our results also indicated that patients with an allergic history, who are challenging to diagnose, may result in false-positive occurrences. Additionally, the PPV is reflective of our population (potential EE patients) that was composed of about 50% non-EE and 50% EE in our cohort. In the general population, where the prevalence of EE is lower, the PPV would thus be lowered. While the cytokine dysregulation was not reproduced in the prospective study, specificity and sensitivity were relatively high due to the high threshold levels chosen, which were set above the maximum level observed in the non-EE group.

It is tempting to speculate on the potential roles of the cytokines that were significantly modified in EE compared to NL. CXCL14 downregulation has also been shown in squamous head and neck cancer and has an anti-proliferative role on epithelial cells. It is possible that specific EGFR tyrosine kinase inhibitor, which restores CXCL14 expression in head and neck squamous cell carcinoma,(26;27) contributes to a decrease in esophageal epithelial cell proliferation in EE patients. In contrast, *CCL23* mRNA is increased in EE and has been shown to be induced following STAT-6 activation:(28) CCL23 is involved in endothelial cell proliferation, a feature that may contribute to the papillae elongation observed in EE. We have also identified dysregulation of novel cytokines and receptors in EE that deserve further consideration. Additionally, we noted marked changes in IL-1

family related molecules with upregulation of *IL1B* and IL-1 related family member 6 and downregulation of the inhibitory receptor (*IL1RA*) and IL-1 related family member 9. Thus, we propose that EE involves coordinate pro-inflammatory signals triggered by IL-1-related molecules, implying the importance of post IL-1-receptor signaling (e.g. NFkB). Indeed, the EE transcriptome has evidence for activation of this pathway via overexpression of *IL8*, *MCP2*, and *TNFAIP6*.(14)

In conclusion, we have extended our prior knowledge of the molecular pathogenesis of EE by identifying esophageal overexpression of a panel of chemokines and cytokines additional to the previously reported *IL13* and eotaxin-3. Although our screening array encoded for 84 relevant mRNAs, only ~20% were dysregulated in EE. Notably, we identify a strong correlation between *IL13*, *IL5*, and eotaxin-3, but not *IL4* mRNA levels, consistent with the presence of an IL-13-producing Th2 cell population. Using molecular analysis of only eotaxin-3 in a large cohort of patients, ~ 90% sensitivity for diagnosis is obtained. Furthermore, blood levels of the simplistic panel of 8 cytokines reached moderate specificity and sensitivity regardless of the global increase of these cytokines in the different groups of patients; atopy was a confounder for systemic cytokine levels. Taken together, evidence is presented that *IL13* and *IL5* associate with eosinophil and eotaxin-3 levels, indicating the key role of adaptive Th2 immunity in regulating eotaxin-3-driven esophageal eosinophilia in the absence of a consistent systemic change in cytokines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

EE	Eosinophilic Esophagitis
NL	Normal
GERD	Gastroesophageal Reflux Disease
EGFR	Epithelial Growth Factor Receptor
Th2	T helper Type 2
EDN	Eosinophil Derived Neurotoxin

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Figure 1. Cytokine mRNA levels in EE patients

Cytokine mRNA levels were quantified using real-time PCR in 11 NL and 11 EE patients. Expression levels were normalized to the housekeeping gene *GAPDH* and are expressed as a relative ratio. EE patients were subdivided based on the maximum eosinophil number in the biopsy into active (> 23 eosinophils/hpf), intermediate (partially treated EE with $1 \leq 23$ eosinophils/hpf), and inactive (0 eosinophils/hpf). *p* values were calculated using the Mann-Whitney test: **p* < 0.05, ***p* < 0.005, and ****p* < 0.0005.

Figure 2. Eotaxin-3, *IL5*, *IL4*, *IL13* and *IL5RA* mRNA levels in EE patients

Levels were quantified using real-time PCR in a large cohort of NL, CE and EE patients. Expression levels were normalized to the housekeeping gene *GAPDH* and are expressed as a relative ratio. EE patients were subdivided based on the maximum eosinophil number in the biopsy into active (> 23 eosinophils/hpf), intermediate (partially treated EE with $1 \leq 23$ eosinophils/hpf), and inactive (0 eosinophils/hpf). *p* values were calculated using the Kruskal-Wallis test with a Dunn's Multiple Comparison Test: **p* < 0.05, ***p* < 0.005, ****p* < 0.0005.

Figure 3. *IL4*, *IL5*, *IL13*, *IL5RA*, and eotaxin-3 expression in active allergic and active non-allergic EE patients

IL4, *IL5*, *IL13*, *IL5RA*, and eotaxin-3 mRNA levels were quantified using real-time PCR in active EE patients (> 23 eosinophils/hpf) from the large cohort. Expression levels were normalized to the housekeeping gene *GAPDH* and are expressed as a relative ratio. *p* values were calculated using the Mann-Whitney test: **p* < 0.05, ***p* < 0.005, ****p* < 0.0005.

Figure 4. Correlation and linear regression between IL4, IL5, IL13, and eotaxin-3
 IL4, IL5, IL13, and eotaxin-3 mRNA levels were quantified using real-time PCR in active EE patients. Spearman correlations r (italics) and p values were calculated to test correlation between the ranked cytokine levels (nonparametric). Linear regression curves are presented with the corresponding p value and r^2 (bold) on the curves to test if the levels of cytokine directly correlate with each other. A non-significant p value (ns) indicates that the slope of the curve is not significantly different from 0.

Figure 5. Plasma cytokine levels in NL and EE patients

Blood cytokine levels significantly differentially expressed in NL and EE patients are shown for IL-4, IL-5, IL-6, IL-8, IL-17, IL-13, IL-12p70, CD40L, and IL-1 α (A). Blood cytokine levels in EE patients before and after therapy are shown (B). The p values were calculated using the Mann-Whitney test (A) or paired t test (B): * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

Table 1

Cytokines with a greater than 4-fold change in expression in EE compared to control group or with a p value < 0.05

Symbol	Discovery Cohort PCR Array ¹	Replication Cohort Real-time PCR ²	Fold Change	p value	Fold Change	p value	Description
<i>CCL26</i>	69.4909	0.00117	66.56	< 0.0001	66.56	< 0.0001	Chemokine (C-C motif) ligand 26 (eotaxin-3)
<i>ABCF1</i>	18.4482	0.297024	5.75	< 0.0001	5.75	< 0.0001	ATP-binding cassette, sub-family F, member 1
<i>CXCL1</i>	16.2004	0.017537	15.87	0.0012	15.87	0.0012	Chemokine (C-X-C motif) ligand 1 (GRO-alpha)
<i>CCL23</i>	7.3190	0.188826	7.19	0.0003	7.19	0.0003	Chemokine (C-C motif) ligand 23 (MIP-3)
<i>IL1B</i>	7.2917	0.073535	5.52	0.0001	5.52	0.0001	Interleukin 1, beta
<i>IL8</i>	6.8396	0.100458	5.75	0.0084	5.75	0.0084	Interleukin 8
<i>CCL8</i>	6.5164	0.243579	5.412	0.052	5.412	0.052	Chemokine (C-C motif) ligand 8 (MCP-2)
<i>IL1F9</i>	6.3033	0.08591	3.09 ³	0.38	3.09 ³	0.38	Interleukin 1 family, member 9
<i>CD40LG</i>	5.7611	0.234128	ND ⁴	ND ⁴	ND ⁴	ND ⁴	CD40 ligand
<i>CXCL2</i>	5.5601	0.244344	18.67	0.0002	18.67	0.0002	Chemokine (C-X-C motif) ligand 2 (GRO-beta, MIP-2)
<i>CXCL3</i>	5.4882	0.344369	ND ⁴	ND ⁴	ND ⁴	ND ⁴	Chemokine (C-X-C motif) ligand 3 (GRO-gamma, MIP-2b)
<i>CCR5</i>	5.2325	0.396277	ND ⁴	ND ⁴	ND ⁴	ND ⁴	Chemokine (C-C motif) receptor 5
<i>IL5RA</i>	4.9223	0.358758	>49.14 ⁵	0.0001	>49.14 ⁵	0.0001	Interleukin 5 receptor, alpha
<i>CCL1</i>	4.8359	0.355615	5.28	0.001	5.28	0.001	Chemokine (C-C motif) ligand 1 (TCA3)
<i>IL9</i>	4.7521	0.380627	5.61 ³	0.7621	5.61 ³	0.7621	Interleukin 9
<i>IL17C</i>	4.6566	0.244893	ND ^{3,4}	ND ⁴	ND ^{3,4}	ND ⁴	Interleukin 17C (CX2)
<i>BCL6</i>	4.4645	0.174119	3.56	0.0126	3.56	0.0126	B-cell CLL/lymphoma 6
<i>IL13</i>	4.2122	0.360454	15.87	< 0.0001	15.87	< 0.0001	Interleukin 13
<i>CCL20</i>	4.0105	0.251954	6.03	0.0432	6.03	0.0432	Chemokine (C-C motif) ligand 20 (MIP-3a)
<i>IL1RN</i>	-2.6497	0.011341	-4.03	0.0002	-4.03	0.0002	Interleukin 1 receptor antagonist (IL-1Ra)
<i>IL1F6</i>	-4.5249	0.067208	-9.92	0.0001	-9.92	0.0001	Interleukin 1 family, member 6 (epsilon)
<i>CXCL14</i>	-9.2850	0.062609	-5.67	0.00287	-5.67	0.00287	Chemokine (C-X-C motif) ligand 14 (BRAX)

¹ Discovery cohort n = 5 NL and n = 5 EE

² Replication cohort n = 11 NL and n = 11 EE

³ Levels were undetectable in several patients in both groups

⁴ Not done or not reproducible

⁵ Levels were not detectable in several NL patients

Table II

Scoring of the 36 blinded samples, diagnosis, allergic history, and peak eosinophil counts in esophageal biopsies

	IL-1a	IL-4	IL-5	IL-6	IL-12 p70	IL-13	IL-17	CD40L	Score	EE	Allergy	Eos #
1	0	0	0	0	1	0	1	0	2	Yes	Yes	207
2	0	0	1	0	1	0	1	1	4	Yes	No	78
3	0	0	1	0	1	0	1	0	3	Yes	No	50
4	0	0	1	0	1	0	0	1	3	Yes	No	267
5	0	0	1	0	0	0	1	0	2	Yes	Yes	169
6	0	0	0	0	0	0	0	1	1	No	Yes	0
7	0	0	0	0	1	0	0	1	2	No	No	0
8	0	0	0	0	0	0	0	1	1	No	No	0
9	0	0	0	0	0	0	0	1	1	No	No	0
10	0	0	0	0	1	0	0	1	2	No	Yes	0
11	0	0	0	0	1	0	0	1	2	No	Yes	0
12	0	0	0	0	0	0	0	1	1	No	Yes	0
13	0	0	0	0	1	0	1	1	3	Yes	Yes	194
14	0	0	1	0	1	0	0	1	3	Yes	Yes	186
15	0	0	0	0	1	0	1	1	3	Yes	Yes	40
16	0	0	0	0	0	0	0	1	1	Yes	Yes	106
17	0	0	1	0	1	0	1	1	4	Yes	Yes	27
18	0	0	0	0	1	0	1	0	2	Yes	No	29
19	0	0	1	0	1	0	1	1	4	Yes	Yes	154
20	0	0	0	0	1	0	1	1	3	Yes	Yes	97
21	0	0	0	0	1	0	0	1	2	No	No	0
22	0	0	0	0	1	0	1	0	2	Yes	Yes	81
23	0	0	0	0	1	0	1	1	3	Yes	Yes	136
24	0	0	1	0	0	0	0	1	2	Yes	Yes	169
25	0	0	0	0	1	0	0	1	2	No	Yes	0
26	0	0	0	0	1	0	1	1	3	No	Yes	0
27	0	0	0	0	1	0	1	1	3	No	Yes	2
28	0	0	1	0	0	0	0	1	2	No	Yes	0

	IL-1a	IL-4	IL-5	IL-6	IL-12 p70	IL-13	IL-17	CD40L	Score	EE	Allergy	Eos #
29	0	0	0	0	1	0	1	1	3	Yes	Yes	105
30	0	0	0	0	1	0	0	1	2	No	No	0
31	0	0	1	0	1	0	1	1	4	No	Yes	0
32	0	0	1	0	0	0	1	0	2	Yes	Yes	103
33	0	0	0	0	1	0	0	1	2	No	Yes	0
34	0	1	1	0	0	0	0	0	2	No	Yes	0
35	0	0	0	0	1	0	1	0	2	No	No	0
36	0	0	0	0	0	0	0	1	1	No	Yes	0

EE, eosinophilic esophagitis
Eos #, maximum eosinophil count / hpf

Table III

Assessment of the specificity and sensitivity of the test

Test results		EE diagnosis		
		Positive n = 18	Negative n = 18	
	Positive n = 14	TP = 11	FP = 3	
	Negative n = 22	FN = 7	TN = 15	
		Sensitivity=61%	Specificity=83%	

TP = true positive

FP = false positive

TN = true negative

FN = false negative

PPV = positive predictive value = $TP/(TP+FP)$ NPV = negative predictive value = $TN/(FN+TN)$ Sensitivity = $TP/(TP+FN)$ Specificity = $TN/(TN+FP)$