Rapid publication

IL-13 involvement in eosinophilic esophagitis: Transcriptome analysis and reversibility with glucocorticoids

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Background: Eosinophilic esophagitis (EE) is an emerging worldwide disease that mimics gastroesophageal reflux disease. Early studies have established that esophageal eosinophilia occurs in association with T_H2 allergic responses, and we recently identified an EE-specific esophageal transcriptome that included eotaxin-3.

Objective: We sought to determine the mechanism by which this T_H2 response leads to EE.

Methods: Real-time PCR and microarray analysis were performed on RNA extracted from esophageal biopsy specimens and primary esophageal epithelial cell cultures stimulated with IL-13 (0-100 ng/mL). Transient transfections in esophageal cell lines were performed with plasmids containing the luciferase gene driven by eotaxin-3 promoter fragments and modified forms of signal transducer and activator of transcription 6.

Results: The IL-13 mRNA level was markedly increased (16-fold) in esophageal biopsy specimens from patients with EE compared with those from healthy individuals. Furthermore, IL-13 treatment of primary esophageal epithelial cells was sufficient to induce a global-expression transcript profile that remarkably overlapped with the EE-specific esophageal transcriptome. In addition, esophageal epithelial cells markedly produce eotaxin-3 after IL-13 stimulation through a transcriptional mechanism dependent on signal transducer and activator of transcription 6. Lastly, increased IL-13 mRNA levels and the EE transcriptome were largely reversible with glucocorticoid treatment *in vivo*.

Conclusions: Taken together, we propose that the pathogenesis of EE is mediated by an IL-13–stimulated keratinocyte-derived transcriptome that is largely reversible with corticosteroid treatment. Furthermore, we identify an *in vivo* IL-13–induced transcriptome that has potential utility for target assessment after anti-IL-13 therapeutics.

Clinical implications: IL-13–induced pathways and genes are fundamental processes in the cause and manifestations of EE; as such, therapeutic agents that interfere with IL-13 might be particularly useful for disease treatment. (J Allergy Clin Immunol 2007;120:1292-300.)

Key words: *IL-13, eosinophilic esophagitis, eotaxin-3, cytokines, esophageal epithelial cells, keratinocytes*

Eosinophilic esophagitis (EE) is an emerging worldwide disease that mimics gastroesophageal reflux disease and can lead to esophageal narrowing and stricture.[1] EE is differentiated from gastroesophageal reflux disease by the lack of response to acid suppression, the magnitude of mucosal eosinophilia and epithelial thickening, its male predominance, and a high rate of association with atopy.[2-4]

The T_H2 cell–derived cytokines IL-4 and IL-13 are produced at increased levels in allergic tissue and are thought to be central regulators of many of the hallmark features of eosinophilic disease.[5] Although some preliminary investigations have suggested that circulating T cells and eosinophils overproduce IL-13 in patients with EE,[6,7] the production of IL-13 by esophageal cells, including

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Supported in part by National Institutes of Health grants AI070235 and AI45898 (M.E.R.), the Food Allergy and Anaphylaxis Network (M.E.R.), the Campaign Urging Research for Eosinophil Disorders, the Buckeye Foundation (M.E.R.), the Food Allergy Project (M.E.R.), the American Heart Association 0625296B (C.B.), the Thrasher Research Funds NR-0014 (C.B.), the Instituto Carlos III Fondo de Investigación Sanitaria CD05/00060 (M.V.), Public Health Service grant CA102357 (S.I.W.), the Translational Research Initiative, Cincinnati Children's Hospital Medical Center, and the DHC (NIDDK 064403).

Disclosure of potential conflict of interest: M. E. Rothenberg has consulting arrangements with Ception Therapeutics and Merck, owns stock in Ception Therapeutics, and is on the speakers' bureau for Merck. The rest of the authors have declared that they have no conflict of interest.

Transcript profiling is available online at http://cypher.cchmc.org:1104. The reader should login as a guest and select "HG-U133 genome"; experiments and gene lists are located in the folder named MRothenberg/blanchard et al.

Received for publication October 3, 2007; revised October 12, 2007; accepted for publication October 15, 2007.

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0091-6749/$32.00

doi:10.1016/j.jaci.2007.10.024

Abbreviations used
EE: Eosinophilic esophagitis
ER: Estrogen receptor
FP: Fluticasone propionate
STAT6: Signal transducer and activator of transcription 6

infiltrating cells, has not been examined. The mechanisms by which IL-13 mediates its effects vary between tissues[5,8,9] because IL-13 induces dramatically different transcriptional profiles in different cell types.[10-12]

All 3 human eotaxin promoters contain signal transducer and activator of transcription 6 (STAT6) consensus-binding motifs, but their relative importance and mode of action differ.[13-18] Interestingly, patients with EE have an esophageal transcriptome characterized by differential expression of 574 genes that is remarkably conserved between individuals, despite their atopic status or sex.[19] Notably, eotaxin-3 is the most overexpressed gene within the EE transcriptome (53-fold increase compared with healthy individuals), even though eotaxin-1 and eotaxin-2 are not significantly induced in patients with EE.

In this study we aimed to uncover the molecular mechanisms involved in the development of EE by focusing on the signaling pathway responsible for the induction of the EE-specific transcriptome. Our data frame the theory that the pathogenesis of EE is mediated by an IL-13–stimulated keratinocyte-derived transcriptome that is largely reversible with corticosteroid treatment. In addition, we have defined an *in vivo* IL-13–associated gene signature with potential value for efficacy assessment of anti-IL-13 therapeutics that are under active clinical testing.

METHODS

Cell culture

A human esophageal adenocarcinoma cell line and squamous epithelial cells (TE1, TE6, TE7, and TE13) were provided by Dr Hainault (IARC, Lyon, France). These cell lines, originally selected from esophageal tumors and well characterized by Nishihira et al,[20,21] were maintained in RPMI medium (Invitrogen, Carlsbad, Calif) supplemented with 10% FCS and 1% penicillin/streptomycin/amphotericin (Invitrogen). For primary epithelial cell culture, the culture conditions and cytokine treatments are provided in the Methods section in the Online Repository (available at www.jacionline.org). This study was approved by the Institutional Review Board of the Cincinnati Children's Hospital Medical Center.

DNA microarray analysis

RNA extraction, microarray analysis, and ontological assessment were performed as previously described.[19] For identification of the resistant genes, the EE transcriptome gene list was applied to fluticasone propionate (FP)–treated patients, and genes significantly expressed differently ($P \leq .05$) between FP-treated and healthy patients were subjected to fold-change filter (≥ 2). Response to therapy was defined as 1 or fewer eosinophil per high-powered field and no epithelial cell hyperplasia after at least 3 months' treatment with FP; these sets of patients are extensions of the analysis done previously.[22]

Reporter constructs, expression vectors, and transient transfections

Plasmids were constructed or obtained and cells were transfected, as described previously.[13] Approximately 10^4 cells per well (24-well plates) were plated on day 1 and transfected with Lipofectamine reagent (Invitrogen), as previously described.[13]

Flow cytometric analysis

Flow cytometric analysis of interleukin receptor chains (human IL-4Rα, human IL-13Rα1, and human IL-13Rα2) was performed as previously described.[13] Briefly, the TE-7 cell line was incubated with 1 μg of mAbs, anti-human IL-4Rα, anti-human IL-13Rα1 (mAb IgG1; Diaclone, Stamford, Conn), or anti-human IL-13Rα2 or IgG1 isotype and 0.4 μg of fluorescein isothiocyanate–labeled secondary antibody. Cells were then analyzed for fluorescence by using single-color flow cytometry with a flow cytometer FACScalibur and analyzed with FlowJo software (TreeStar, Inc, Ashland, Ore).

ELISA protocol for IL-13, eotaxin-1, eotaxin-2, and eotaxin-3 quantification

Eotaxin-1, eotaxin-2, and eotaxin-3 Duo set ELISAs were performed according to the manufacturer's instruction (R&D Systems, Minneapolis, Minn), as previously described.[19,23] The IL-13 Quantikine Kit (R&D Systems) was used to quantify IL-13 protein levels. The detection limits were 70, 200, 200 and 62 pg/mL for eotaxin-1, eotaxin-2, eotaxin-3, and IL-13, respectively.

Statistical analysis

Data are expressed as means ± SD. Statistical significance comparing 2 different treatments or groups was determined by using the Student *t* test (normal distribution equal variance), the Welch *t* test (normal distribution unequal variances), the Mann-Whitney test (nonparametric test, 2 groups), or the Kruskal-Wallis test, followed by a Dunn multiple comparison test (nonparametric test, ≥ 3 groups) with Prism 4 Software.

RESULTS

IL-13 expression in patients with EE

To establish the participation of IL-13 (and/or IL-4) in EE pathogenesis, we wanted first to establish whether IL-13 was overproduced in the esophageal tissue of patients with EE. By using real-time PCR analysis, there was a 16-fold increase in IL-13 mRNA in patients with EE compared with that seen in healthy patients (defined as individuals with no gastrointestinal pathology) (Fig 1, *A*). Interestingly, the IL-4 mRNA level was not significantly increased in our patients with EE compared with that seen in healthy patients, although a subgroup of patients showed an increase in IL-4 levels (Fig 1, *B*). Notably, there was a correlation between IL-13 mRNA and eotaxin-3 expression ($r^2 = 0.49$; $P < .05$).

IL-13–induced esophageal epithelial genes

Primary esophageal epithelial cells from the esophagi of patients with EE were cultured and stimulated with 100 ng/mL IL-13 for 48 hours to identify IL-13–induced esophageal epithelial genes. The mRNA was subjected to global transcript-expression profile analysis and normalized pairwise to unstimulated controls. Genes were filtered to fold change in biopsy specimens from patients with EE

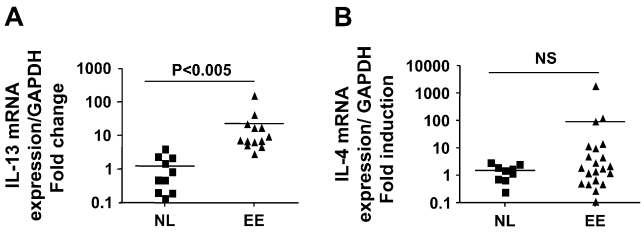


FIG 1. IL-13 and IL-4 mRNA expression in biopsy samples from healthy *(NL)* subjects and patients with EE. The expression of IL-13 **(A)** and IL-4 **(B)** is shown. Each mRNA value is normalized to glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* mRNA expression from the same sample and is expressed as a fold increase. The *black lines* represent the mean value in each group. *P* values were calculated by using the Mann-Whitney *U* test (2 groups; n = 8-9 and 13-21 subjects for the healthy and EE groups, respectively).

compared with those from healthy patients and IL-13–stimulated epithelial cells. A highly significant positive correlation ($P < .0001$, Spearman) between modified genes in biopsy specimens from patients with EE and in IL-13–stimulated keratinocytes was observed (Fig 2, *A*). Eotaxin-3, cadherin-26, and TNF-α–induced protein 6 were upregulated in both specimens from patients with EE and IL-13–stimulated keratinocytes. Mast cell genes (carboxypeptidase A3), eosinophil genes (Charcot Leyden Crystal protein) and lymphocyte genes (immunoglobulin chains) were upregulated more than 10-fold in patients with EE compared with values in healthy patients[19] but were not upregulated in IL-13–stimulated epithelial cells (Fig 2, *A*). In IL-13–stimulated cells, 4698 and 952 genes were significantly modified by using different stringencies of statistical comparisons ($P < .05$ and $P < .01$, respectively) compared with unstimulated cells. There were 1333 genes modified by more than 1.5-fold (780 upregulated and 553 downregulated) and 371 genes modified by 2-fold or greater (255 genes were upregulated and 116 were downregulated; Fig 2, *B* and *C*, and Table E1 in the Online Repository at www.jacionline.org). Ontological analysis of the 780 upregulated genes revealed that the most significant biologic processes involved pathways that regulated cell cycle ($P = 7.04 \times 10^{-6}$), response to external stimulus ($P = 1.33 \times 10^{-5}$), response to wounding ($P = 1.77 \times 10^{-5}$), and cell proliferation ($P = 2.90 \times 10^{-5}$). In contrast, downregulated genes were involved in ectoderm development ($P = 3.01 \times 10^{-7}$), epidermis development ($P = 7.28 \times 10^{-7}$), tissue development ($P = 2.53 \times 10^{-5}$), and keratinization ($P = 2.63 \times 10^{-5}$). Genes that were upregulated 5-fold or greater and downregulated 4-fold or greater in primary esophageal keratinocytes stimulated with IL-13 (100 ng/mL, 48 hours) are shown in Fig 2, *B*.

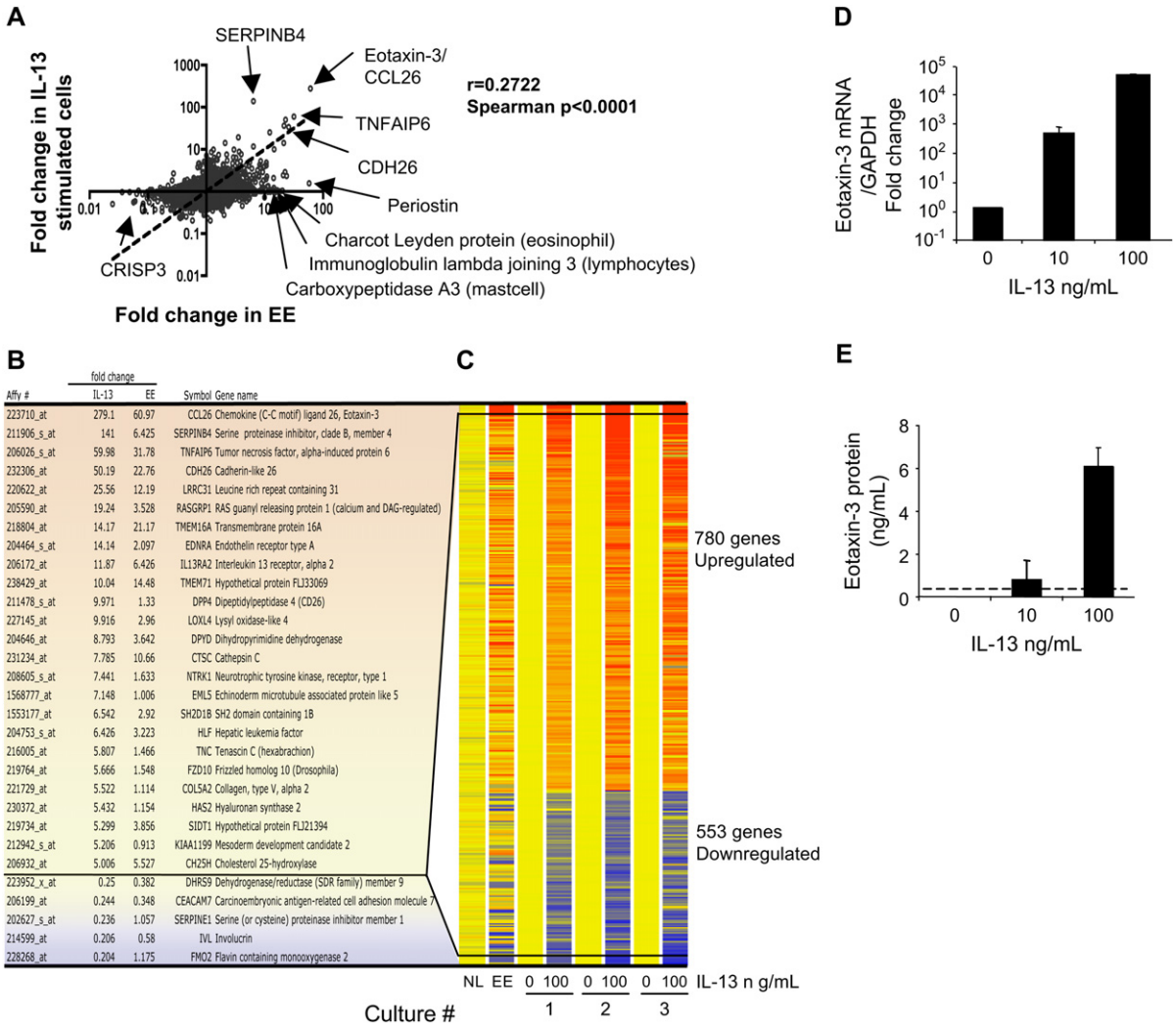
We were interested in determining whether the IL-13–regulated gene signature overlapped with the EE transcriptome.[19] We therefore compared the IL-13–induced gene transcript profile with the EE-specific transcriptome (Fig 2, *A-C*). Notably, 126 of the IL-13–induced genes (22% of the EE transcriptome, $P < .05$) overlapped with the EE transcriptome. Interestingly, the number one gene overexpressed in IL-13–stimulated keratinocytes was eotaxin-3, which was upregulated 279-fold (Fig 2, *B*). Remarkably, eotaxin-1 and eotaxin-2 were not overexpressed in IL-13–treated primary keratinocyte cultures.

IL-13–induced eotaxin-3 expression *ex vivo*

Primary keratinocytes were stimulated with 0 to 100 ng/mL human IL-13 for 48 hours to further test whether IL-13 induces eotaxin-3 mRNA expression in the esophagi of patients with EE. Eotaxin expression was studied by means of real-time PCR (Fig 2, *D*). Eotaxin-3 expression was induced in a dose-dependent manner after IL-13 stimulation. Remarkably, there was a 1000- and 10,000-fold increase of eotaxin-3 mRNA expression 48 hours after 10 and 100 ng/mL IL-13, respectively. Although not detectable at baseline, IL-13 (100 ng/mL) induced a dramatic release of eotaxin-3 protein (Fig 2, *E*) into the supernatant (6.03 ± 0.8 ng/mL), representing at least a 30-fold increase over the detection limit (200 pg/mL). Importantly, eotaxin-1 and eotaxin-2 mRNA and protein were not overexpressed under these conditions (data not shown).

Eotaxin-3 expression in esophageal epithelial cell lines after IL-13 stimulation

To investigate the molecular mechanisms involved in IL-13–induced eotaxin-3 expression in keratinocytes, we examined human esophageal epithelial cell lines (TE-1, TE-6, TE-7, and TE-13). First, by using RT-PCR, we demonstrated that the receptor chains of IL-13 (IL-13Rα1, IL-13Rα2, and IL4Rα) are expressed by these cell lines (Fig 3, *A*). The respective protein products were detected by means of FACS analysis in TE-7 esophageal epithelial cells (Fig 3, *B*). All cell lines were subsequently stimulated with increasing concentrations of human IL-13 for 0 to 48 hours (Fig 3, *C*). Eotaxin-3 mRNA expression was increased in a dose-dependent manner, although to varying degrees between the cell lines. Eotaxin-3 mRNA expression was increased by 4-, 8-, 77-, and 1007-fold in TE-1, TE-6, TE-7, and TE-13 cells, respectively, after IL-13 treatment at 100 ng/mL. Baseline eotaxin-3 protein expression was less than the detection limit of 200 pg/mL but was overexpressed in the cell contents and in the supernatant of IL-13–stimulated TE-7 cells in a dose- and time-dependent fashion (Fig 3, *D* and *E*). After IL-13 stimulation (100 ng/mL for 24 hours), 2.5 ± 0.7 ng/mL and 0.55 ± 0.37 ng/mL eotaxin-3 was released in the supernatants of TE-7 and TE-13 cells, respectively. Notably, eotaxin-1 and eotaxin-2 expression levels were less than or at the detection limit of the real-time PCR and were



Affy #	fold change IL-13	fold change EE	Symbol	Gene name
223710_at	279.1	60.97	CCL26	Chemokine (C-C motif) ligand 26, Eotaxin-3
211906_s_at	141	6.425	SERPINB4	Serine proteinase inhibitor, clade B, member 4
206026_s_at	59.98	31.78	TNFAIP6	Tumor necrosis factor, alpha-induced protein 6
232306_at	50.19	22.76	CDH26	Cadherin-like 26
220622_at	25.56	12.19	LRRC31	Leucine rich repeat containing 31
205590_at	19.24	3.528	RASGRP1	RAS guanyl releasing protein 1 (calcium and DAG-regulated)
218804_at	14.17	21.17	TMEM16A	Transmembrane protein 16A
204464_s_at	14.14	2.097	EDNRA	Endothelin receptor type A
206172_at	11.87	6.426	IL13RA2	Interleukin 13 receptor, alpha 2
238429_at	10.04	14.48	TMEM71	Hypothetical protein FLJ33069
211478_s_at	9.971	1.33	DPP4	Dipeptidylpeptidase 4 (CD26)
227145_at	9.916	2.96	LOXL4	Lysyl oxidase-like 4
204646_at	8.793	3.642	DPYD	Dihydropyrimidine dehydrogenase
231234_at	7.785	10.66	CTSC	Cathepsin C
208605_s_at	7.441	1.633	NTRK1	Neurotrophic tyrosine kinase, receptor, type 1
1568777_at	7.148	1.006	EML5	Echinoderm microtubule associated protein like 5
1553177_at	6.542	2.92	SH2D1B	SH2 domain containing 1B
204753_s_at	6.426	3.223	HLF	Hepatic leukemia factor
216005_at	5.807	1.466	TNC	Tenascin C (hexabrachion)
219764_at	5.666	1.548	FZD10	Frizzled homolog 10 (Drosophila)
221729_at	5.522	1.114	COL5A2	Collagen, type V, alpha 2
230372_at	5.432	1.154	HAS2	Hyaluronan synthase 2
219734_at	5.299	3.856	SIDT1	Hypothetical protein FLJ21394
212942_s_at	5.206	0.913	KIAA1199	Mesoderm development candidate 2
206932_at	5.006	5.527	CH25H	Cholesterol 25-hydroxylase
223952_x_at	0.25	0.382	DHRS9	Dehydrogenase/reductase (SDR family) member 9
206199_at	0.244	0.348	CEACAM7	Carcinoembryonic antigen-related cell adhesion molecule 7
202627_s_at	0.236	1.057	SERPINE1	Serine (or cysteine) proteinase inhibitor member 1
214599_at	0.206	0.58	IVL	Involucrin
228268_at	0.204	1.175	FMO2	Flavin containing monooxygenase 2

FIG 2. Gene expression analysis in primary esophageal cells after IL-13 stimulation and comparison with the EE transcript signature. **A**, The 54,765 genes of the HG-U133 chip were subjected to fold-change filter in patients with EE versus healthy subjects and IL-13–stimulated primary cell cultures versus unstimulated cells. Spearman correlation and linear regression were calculated. **B**, The list displays 33 transcripts that were upregulated 5-fold or greater and 5 transcripts that were downregulated 4-fold or greater compared with unstimulated cells. **C**, The genes modified by 1.5-fold or greater on average in IL-13–stimulated cells (100 ng/mL) are presented in a heat diagram in 3 primary-culture patient biopsy specimens (*1*, *2*, and *3*), unstimulated and stimulated. Upregulated genes are shown in red, and downregulated genes are shown in blue. The magnitude of the gene changes is proportional to the darkness of the color. **D**, The fold increase of eotaxin-3 mRNA expression compared with the untreated value was quantified by means of real-time PCR. **E**, Eotaxin-3 released in the culture supernatant is expressed in nanograms per milliliter. Results are presented as means ± range and are representative of experiments performed in 5 different patients.

not overexpressed in these cell lines after IL-13 stimulation (data not shown).

Eotaxin-3 promoter activity

Activated STAT6 has been shown to bind to consensus sites located at −698 and −89 bp in skin and kidney fibroblasts and epithelial cell lines of the lung and colon.[13-15,24] To determine the STAT6 element or elements involved in the eotaxin-3 stimulation observed in esophageal cell lines, we transiently transfected the TE-7 cell line with the full-length eotaxin luciferase reporter plasmid and truncated versions. There was a significant increase of 2.7 ± 0.8–fold and 10.8 ± 2.2–fold of luciferase activity with the full-length reporter plasmid at IL-13 doses of 10 and 100 ng/mL, respectively (Fig 4, *A*). Similar results were observed in the other TE cell lines (data not shown). The TE-7 cell line was transfected with truncated promoter constructs or with a full-length promoter containing specific mutations within the STAT6-binding sites to map the relevant cis-acting promoter

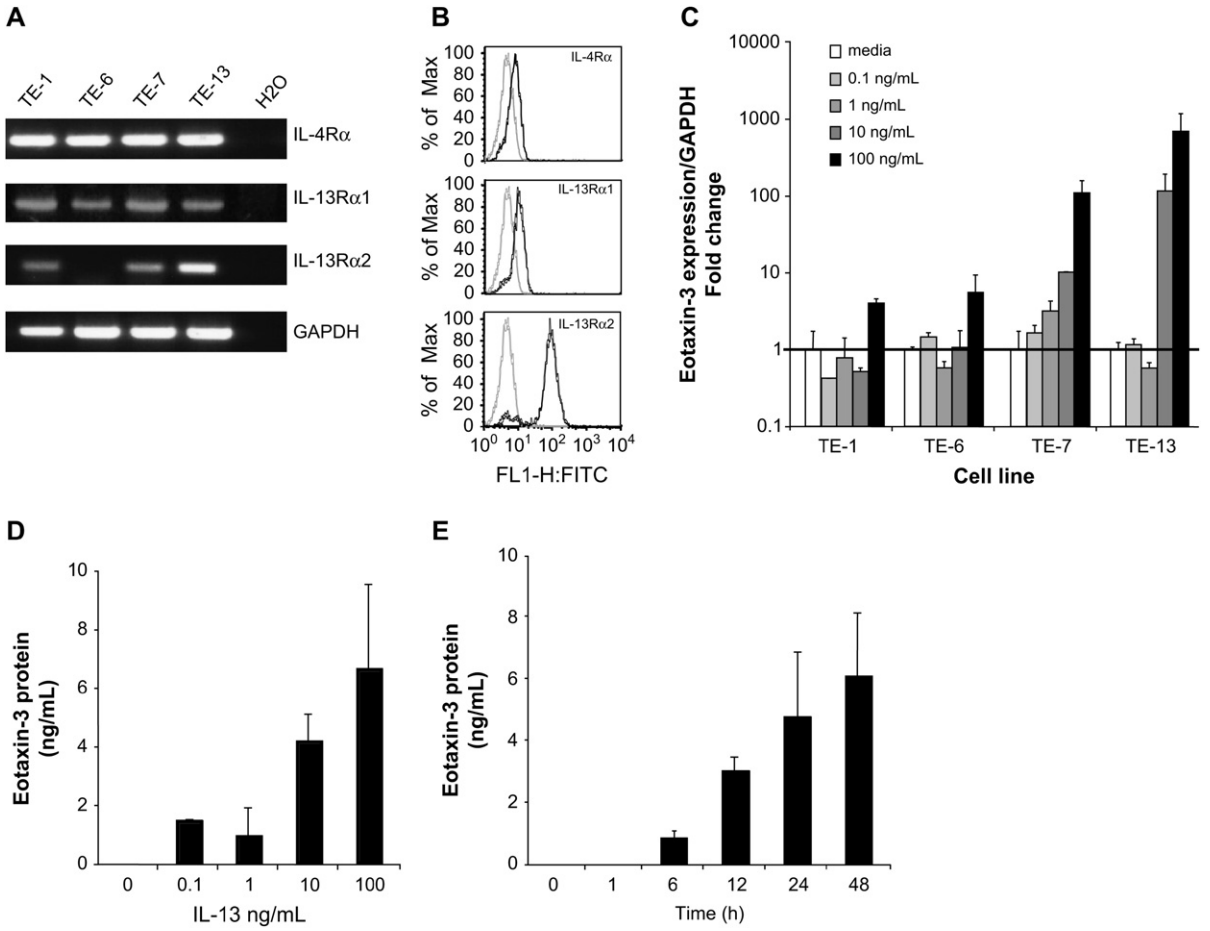


FIG 3. IL-13 receptor chain expression in esophageal cells and eotaxin-3 production by esophageal cell lines after IL-13 stimulation. **A**, The TE-1, TE-6, TE-7, and TE-13 esophageal cell lines were subjected to PCR analysis for IL-4Rα, IL-13Rα1, IL-13Rα2, and glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* mRNA expression. **B**, Flow cytometric analysis of IL-4R, IL-13Rα1, and IL-13Rα2 chain expression in the TE-7 cell line *(dark lines)*. Controls were performed with an irrelevant IgG1 *(gray lines)*. **C**, The TE-1, TE-6, TE-7, and TE-13 esophageal cell lines were stimulated for 24 hours with IL-13 (0, 1, 10, and 100 ng/mL). The fold increase of eotaxin-3 mRNA expression compared with that of the untreated cells is shown. **D** and **E**, TE-7 cells were cultured with IL-13 (0, 1, 10, and 100 ng/mL) for 1, 6, 12, 24, and 48 hours. Eotaxin-3 protein released in the culture supernatant was quantified by means of ELISA. Results are presented as means ± range and are representative of at least 3 experiments performed in triplicate.

sequences (Fig 4, *B*). After IL-13 stimulation, the construct P100 (containing a truncated proximal STAT6 site) had no activity, whereas the promoter containing 800 or 500 bp had full IL-13–induced activity. Furthermore, the construct containing the mutated −89 binding site had no increase in luciferase activity, demonstrating that the proximal STAT6-responsive element at base pair −89 was required for IL-13–induced eotaxin-3 promoter activity.

STAT6-dependent mechanism

Cells were first cotransfected with the eotaxin-3 reporter and a dominant-negative STAT6-expressing vector or empty control vector to definitively implicate STAT6 in eotaxin-3 induction (Fig 4, *C*). The overexpression of the dominant-negative STAT6 dramatically decreased ($P < .05$) activity of the IL-13–induced eotaxin-3 promoter. The cells were also cotransfected with the eotaxin-3 promoter construct and an estrogen-inducible form of STAT6 (fusion between STAT6 and estrogen receptor [ER], STAT6:ER).[25] Subsequently, addition of 4-hydroxytamoxifene to the culture medium (which allows dimerization of the modified ER protein fused to STAT6[26]) resulted in a significant ($P < .01$) induction of the eotaxin-3 promoter luciferase activity within 24 hours (Fig 4, *D*).

Eotaxin-3 mRNA stability

Chemokine mRNA expression can be highly dependent on posttranscriptional mechanisms, such as RNA stability. Experiments with the RNA polymerase II inhibitor actinomycin-D revealed that the stability of eotaxin-3 mRNA was not significantly different between IL-13–treated and nontreated TE-7 cells (Fig 4, *E*), and the half-life of eotaxin-3 in esophageal keratinocytes was determined to

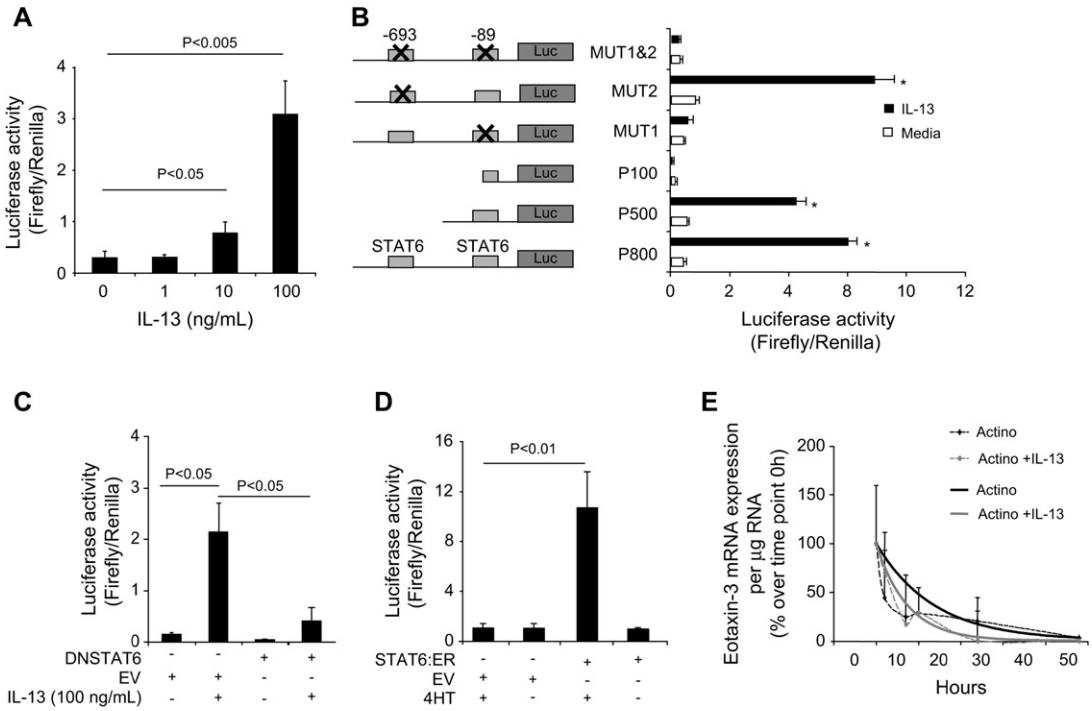


FIG 4. Human eotaxin-3 promoter activity after IL-13 stimulation and the role of STAT6. **A**, TE-7 cells were transfected with pGL3 basic containing the eotaxin-3 promoter (P800) and phRLTK. Cells were stimulated with IL-13 (0, 1, 10, and 100 ng/mL) **B**, TE-7 cells were transfected with pGL3 basic containing different lengths of the eotaxin-3 promoter (*P800*, *P500*, and *P100*) and promoters containing mutations in the STAT6-responsive elements (*MUT1*, *MUT2*, and *MUT1&2*). **C**, The TE-7 cells were cotransfected with P800 and a dominant-negative form of STAT6 *(DNSTAT6)* or the empty vector *(EV)*. **D**, TE-7 cells were cotransfected with P800 and the expression vector containing STAT6:ER. The cells were stimulated with 4-hydroxytamoxifene (*4HT*; 10 μmol/L). Results are presented as the ratio of the luciferase firefly/Renilla activities. **E**, Esophageal keratinocytes (TE-7) were pretreated with IL-13 (0 or 100 ng/mL) and actinomycin D (*Actino*; 0 or 10 μmol/L) for 0 to 48 hours. Results are presented as a percentage of eotaxin-3 mRNA compared with time 0 hours (100%; *black* and *gray dashed lines* for media and IL-13, respectively). Trend lines (*black* and *gray lines* for media and IL-13, respectively) were calculated.

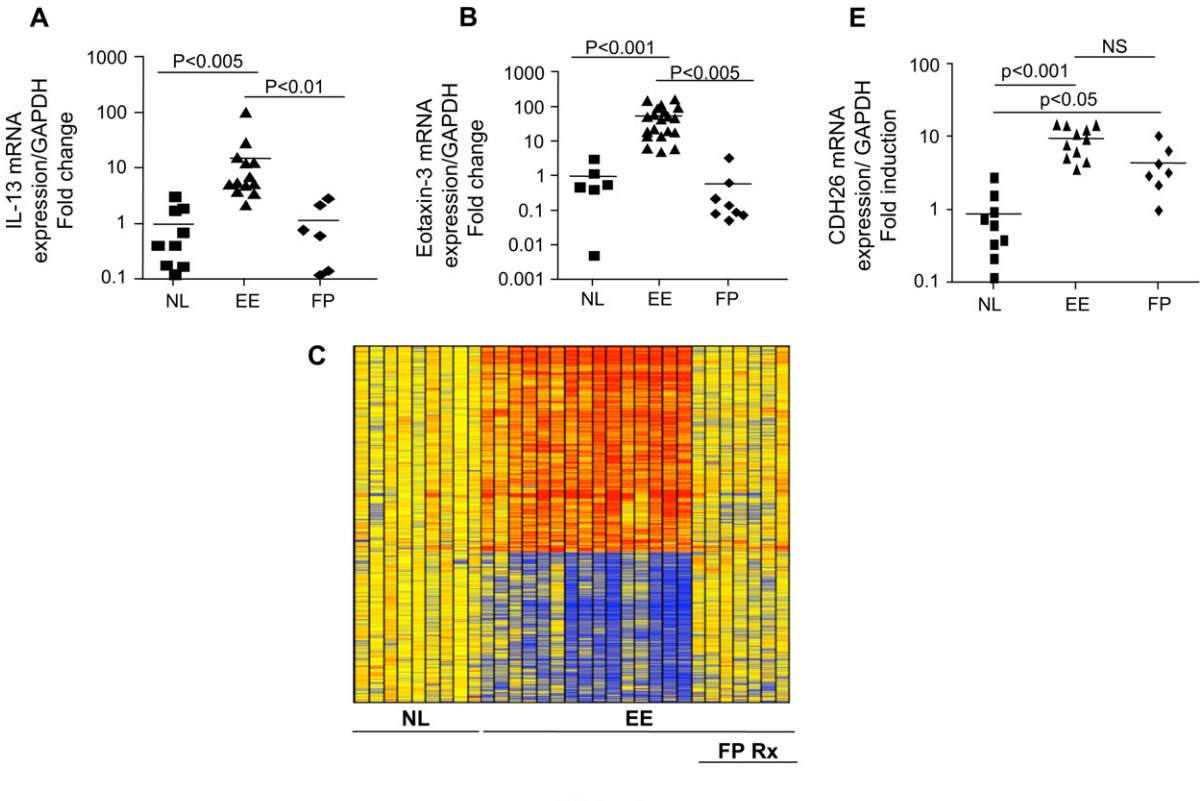
be 5.7 ± 2.3 hours and not significantly modified by IL-13 treatment (Fig 4, *E*). Additionally, the eotaxin-3 3′ untranslated region sequence was subcloned downstream of an SV40 promoter–driven luciferase gene in the pGL3 reporter plasmid. Transfections were performed in the presence or absence of IL-13. No statistically significant increase of luciferase activity after IL-13 stimulation was observed (data not shown).

Identification of glucocorticoid-reversible genes in patients with EE

We have previously reported that FP induces EE disease remission compared with placebo treatment.[22] We aimed to identify whether IL-13 overexpression could be normalized in patients with EE with successful anti-inflammatory intervention. Indeed, IL-13 mRNA levels were significantly ($P < .01$) reduced in EE responders compared with untreated specimens, and expression levels after treatment were comparable with the levels detected in control biopsy specimens (Fig 5, *A*). Similarly, eotaxin-3 mRNA was normalized in FP-responder patients ($P < .01$; Fig 5, *B*). We next aimed to determine whether the EE transcriptome was also reversible in patients with EE successfully treated with glucocorticoids. Remarkably, 98% of the EE transcriptome was reversed to expression levels detected in biopsy specimens of healthy patients (Fig 5, *C*). The reversible genes include cell-specific transcripts, including eosinophil, mast cell, lymphocyte, fibroblast, and epithelial genes. In addition, genes involved in cellular recruitment and cell proliferation were also reversible. Interestingly, although the biopsy specimens had no abnormal microscopic features, 12 genes were still dysregulated (Fig 5, *D*). Among these 12 dysregulated genes, uroplakin and cadherin-26 remained upregulated, and desmoglein remained downregulated. Indeed, real-time PCR demonstrated a residual upregulation of cadherin-26 in FP-treated patients with EE ($P < .05$; Fig 5, *E*).

DISCUSSION

In this study we demonstrated that a large number of EE-associated genes are directly induced by IL-13 in esophageal keratinocytes, and we therefore implicate



D

Affy #	Symbol	Fold change in FP	Fold change in EE	Gene name
210065_s_at	UPK1B	5.826	6.608	Uroplakin 1B
210064_s_at	UPK1B	4.876	5.47	Uroplakin 1B
1553177_at	SH2D1B	2.037	3.367	SH2 domain containing 1B
232306_at	CDH26	4.856	23.22	Cadherin-like 26
233663_s_at	CDH26	3.733	27.41	Cadherin-like 26
219197_s_at	SCUBE2	2.341	6.639	Signal peptide, CUB domain, EGF-like 2
210809_s_at	POSTN	2.471	52.79	Periostin, osteoblast specific factor
203854_at	IF	2.155	6.625	I factor (complement)
236489_at	UNK	2.02	5.838	Transcribed locus
210873_x_at	APOBEC3A	2.382	18.6	Apolipoprotein B mRNA editing enzyme, CPL 3A
210262_at	CRISP2	0.478	0.109	Cysteine-rich secretory protein 2
206642_at	DSG1	0.225	0.133	Desmoglein 1
240420_at	AADACL2	0.439	0.434	Arylacetamide deacetylase-like 2

FIG 5. Effect of glucocorticoids on the EE transcriptome and resistant genes. **A** and **B**, The expression of IL-13 (Fig 5, *A*) and eotaxin-3 (Fig 5, *B*) mRNA is shown in healthy subjects *(NL)*, patients with EE, and patients with EE treated successfully with FP (n = 8-9, 13-19, and 6-8 subjects for the NL, EE, and FP groups, respectively). **C**, Total mRNA was subjected to microarray analysis. Upregulated genes are shown in red, and downregulated genes are shown in blue. Each *column* represents a separate patient (*NL*, *EE*, and *FP Rx*), and each *line* represents a gene. **D**, Genes that are resistant to glucocorticoid therapy are shown with their Affymetrix accession numbers and their fold change in patients with EE and in treated patients with EE. **E**, Expression of cadherin-26 *(CDH26)* was quantified by means of real-time PCR. Each data point corresponds to a separate individual (n = 9, 11, and 7 subjects for the NL, EE, and FP groups, respectively). *P* values were calculated using Kruskal-Wallis tests (3 groups).

IL-13 as a major regulator of the keratinocyte pathways involved in EE. In EE, the esophageal tissue undergoes changes marked by an abnormal accumulation of eosinophils, mast cells,[7,13,27] and lymphocytes[7,13]; epithelial cell hyperplasia; elongation of the papillae (endothelial cells and fibroblasts); and intensive lamina propria remodeling (likely involving fibroblasts).[3,28-32] In our minimalist model system, the stimulation of keratinocytes with IL-13 is able to partially reproduce the EE transcriptome, indicating that this cell type is likely to largely account for the abnormal response seen in endoscopic biopsy specimens. Based on this striking overlap, we support a model in which IL-13–induced gene expression in keratinocytes makes an important contribution to EE. This model, however, does not exclude indirect and paracrine effects.

In a previous study using microarray analysis, IL-4 and IL-13 mRNA were neither detected nor upregulated in patients with EE; however, several other T_H2-inducible molecules were still detected.[19] Using a highly sensitive method (real-time PCR) relative to microarray analysis, we demonstrate that IL-13 mRNA is indeed overexpressed in patients with EE (16-fold). This is consistent with previous studies showing an increase in IL-13 levels in stimulated PBMCs and eosinophils of patients with

EE.[7,33,34] Mast cells, basophils, eosinophils, lymphocytes, and smooth muscle cells are potent IL-13–producing cells[6,7,34,35]; however, the exact cellular source or sources of IL-13 in the esophageal biopsy specimen remains to be elucidated. IL-4 was overexpressed in less than 50% of patients with EE, suggesting a more important involvement for IL-13 in the mucosa for most patients.

In the esophagus, enhanced expression of eotaxin-3 transcript by IL-13 is likely operational through the transcription factor STAT6, with no significant affect on mRNA half-life based on our studies (Fig 4, *E*). Our results are consistent with a recent study in a colonic epithelial cell line[13] but contrast with the literature describing IL-13–induced eotaxin-1 mRNA stabilization in airway epithelial cells; this suggests that different mechanisms can be used for the regulation of distinct eotaxin family members in different cell types.[36] It is notable that the EE transcriptome and IL-13–induced genes do not include eotaxin-1 and eotaxin-2, despite the presence of STAT6-binding sites in both of these genes. Taken together, these results suggest that the regulation of eotaxin-3 occurs differently from that of the other eotaxins and that keratinocytes use a regulatory pathway unique from that of other cells.

Topical FP therapy has been shown to improve clinical symptoms, as well as endoscopic and microscopic features of EE.[22,37,38] In this study we uncover the mechanism involved by demonstrating that successful FP treatment reverses the molecular signature of EE. Although topical glucocorticoids are known to have anti-inflammatory effects, they do not universally reduce levels of all cytokines,[39] and therefore it was important to determine the effect of topical fluticasone on the EE transcriptome. Notably, glucocorticoid treatment was associated with reduced IL-13 and eotaxin-3 production, indicating that the classic features of this T_H2-associated pathway in the esophagus are largely reversible. These reversible genes include cell-specific transcripts from eosinophils, mast cells, lymphocytes, fibroblasts, and epithelial cells, as well as chemoattractants, growth factors, and molecules involved in cell proliferation. These results are consistent with the decrease in eosinophils, mast cells, and epithelial hyperplasia after therapy.[22,37] Interestingly, although the treated biopsy specimens appear microscopically normal, the transcriptome still contains a small number of dysregulated genes. The residual expression of these genes in successfully treated patients with EE suggests that these genes (cadherin-26, desmoglein 1, and uroplakin 1B) might be part of the primary constitutive genetic defect inherent to the epithelium (modifying permeability or elasticity) or have a reduced propensity to respond to glucocorticoid treatment. Of note, eotaxin-3 mRNA and protein levels were only partially decreased by glucocorticoids in IL-13–stimulated esophageal keratinocytes (data not shown). Of interest, the residual expression markers have potential clinical value because they might indeed serve as diagnostic criteria, irrespective of the degree of tissue inflammation (and the expression of the rest of the EE transcriptome). Additionally, these resistant genes might also help in the understanding of the chronic and relapsing nature of the disease.[40]

In summary, our results provide new insight into the molecular pathogenesis of EE. We propose that disease pathogenesis involves a glucocorticoid-reversible IL-13–induced keratinocyte transcriptome that includes eotaxin-3. These results underscore the potential value of new therapeutics interfering with the IL-13/eotaxin-3/CCR3 axis[41]; it is predicted that such agents would not only limit eosinophil accumulation but also the keratinocyte proliferation characteristic of EE. Therapeutic agents that interfere with IL-13 signaling are under active development for asthma. It has not escaped our attention that our newly defined set of IL-13–induced EE transcripts has potential value in the testing of clinical reagents that block IL-13 in patients. Not only do we propose that anti-IL-13 therapeutics be examined in patients with EE because of their potential clinical benefit but also because the EE transcriptome provides a robust means to molecularly monitor drug efficacy and mechanism of action.

We thank Andrea Lippelman for assistance with the preparation of the manuscript, Elisabeth Jones for technical assistance, and Drs Fred Finkelman and Mitchell Cohen for review of the manuscript.

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