From [a]the Institute of Biosciences, University of Sao Paulo State (UNESP), Rio Claro, Sao Paulo, Brazil; [b]Immunological Biotechnology, Department of Engineering, Aarhus University, Aarhus, Denmark; [c]Euroimmun AG, Lübeck, Germany; [d]the Laboratory of Translational Immunology, Faculty of Medicine, University of Campinas (UNICAMP), Campinas, Sao Paulo, Brazil; and [e]the Department of Dermatology and Allergology, University Medical Center Giessen and Marburg, Justus-Liebig University Giessen, Giessen, Germany. E-mail: e.spillner@eng.au.dk.

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Increased GATA-3 and T-bet expression in eosinophilic esophagitis versus gastroesophageal reflux disease

To the Editor:

Eosinophilic esophagitis (EoE) is a clinicopathological condition characterized by symptoms of esophageal dysfunction and dense eosinophil infiltration of the esophageal epithelium. The current diagnostic metric requires 15 eosinophils (eos) per hpf in at least 1 mucosal biopsy specimen following 6 to 8 weeks of treatment with high-dose proton pump inhibitor (PPI).[1] Although this histologic threshold distinguishes most subjects with EoE, several shortcomings exist including the following: (1) eosinophilia may underestimate the extent of eosinophil activity; (2) some patients with gastroesophageal reflux disease (GERD) may have distal esophageal eosinophilia exceeding 15 eos/hpf; and (3) patients often require more than 1 biopsy to determine the underlying diagnosis and establish appropriate treatment. Recent evidence suggests that EoE and proton pump inhibitor-responsive esophageal eosinophilia (PPI-REE) have a similar transcriptome[2] and are phenotypically indistinguishable.[3] Novel histologic biomarkers may further aid in distinguishing causes of esophageal eosinophilia.

Characterizations of mucosal inflammatory responses in EoE and PPI-REE describe a T_H2-predominant pattern with overexpression of T_H2-associated genes.[4] In EoE, IL-5 and IL-13 levels are associated with eotaxin-3 expression, which likely drives eosinophil recruitment. In contrast, esophageal biopsies from patients with GERD are more commonly associated with a T_H1 phenotype defined by increased mRNA expression of IL-1β, IL-8, and IFN-γ.[5]

T-bet and GATA-3 are transcriptional regulators that drive differentiation of T_H0 $CD4^+$ lymphocytes to T_H1 and T_H2 lineages, respectively. We previously demonstrated the utility of characterizing tissue-specific immune polarization using immunohistochemistry-based assessments of GATA-3 and T-bet in bladder cancer.[6] Given its role in T_H2-associated inflammation, we hypothesized that GATA-3 expression would be increased in EoE and PPI-REE and that the ratio of GATA-3/T-bet expression would differentiate these individuals from subjects with GERD.

We performed a retrospective, case-control study of children characterized clinically as having EoE (n = 24), PPI-REE (n = 10), or GERD (n = 28) and as controls (n = 32). Subjects diagnosed with EoE were treated with an elimination diet (n = 7), swallowed topical steroids (n = 12), or a combination of elimination diet and swallowed topical steroids (n = 5) and those with PPI-REE were treated with high-dose PPI (2 mg/kg/d). All subjects with EoE and PPI-REE demonstrated histologic resolution of esophageal eosinophilia (<15 eos/hpf) after 6 to 8 weeks of treatment, respectively.

Tissue sections from active and matched posttreatment biopsies were assessed with hematoxylin and eosin, and immunohistochemical staining for T-bet and GATA-3 was performed. Slides stained for T-bet and GATA-3 were digitized and staining of the epithelial layer was quantified (see Fig E1 in this article's Online Repository at www.jacionline.org). A nuclear algorithm was used to identify T-bet+ and GATA-3+ cells. The number of positive cells was divided by the total area of esophageal epithelium analyzed in order to normalize the number of T-bet+ and GATA-3+ cells/mm.^{2} Polarization of the immune microenvironment was assessed by the GATA-3+ cells/mm^2/T-bet+ cells/mm^2 (G/T) ratio. Details regarding the study population, methods for T-bet/GATA-3 staining/quantification, and statistical analysis are detailed in this article's Online Repository at jacionline.org.

Table E1 in this article's Online Repository at www.jacionline.org details the demographic and clinical characteristics of the study population. Staining for GATA-3 and T-bet by immunohistochemistry is shown in Fig 1. In comparison to GERD, subjects with active EoE demonstrated increased GATA-3+ cells/mm^2 (median, 2.81 vs 8.46 cells/mm^2; $P < .0001$; area under the curve, 0.88; 95% CI, 0.78-0.97) and T-bet+ cells/mm^2 (median, 7.12 vs 12.01 cells/mm^2; $P < .0001$; area under the curve, 0.79; 95% CI, 0.66-0.93) (see Fig 2, *B*, *C*, and *F*). No statistical differences in GATA-3 and T-bet expression were found between subjects with active EoE and PPI-REE. GATA-3 and T-bet expression was also significantly elevated in subjects with active EoE compared with healthy controls. Following treatment, GATA-3 and T-bet expression decreased significantly in subjects with

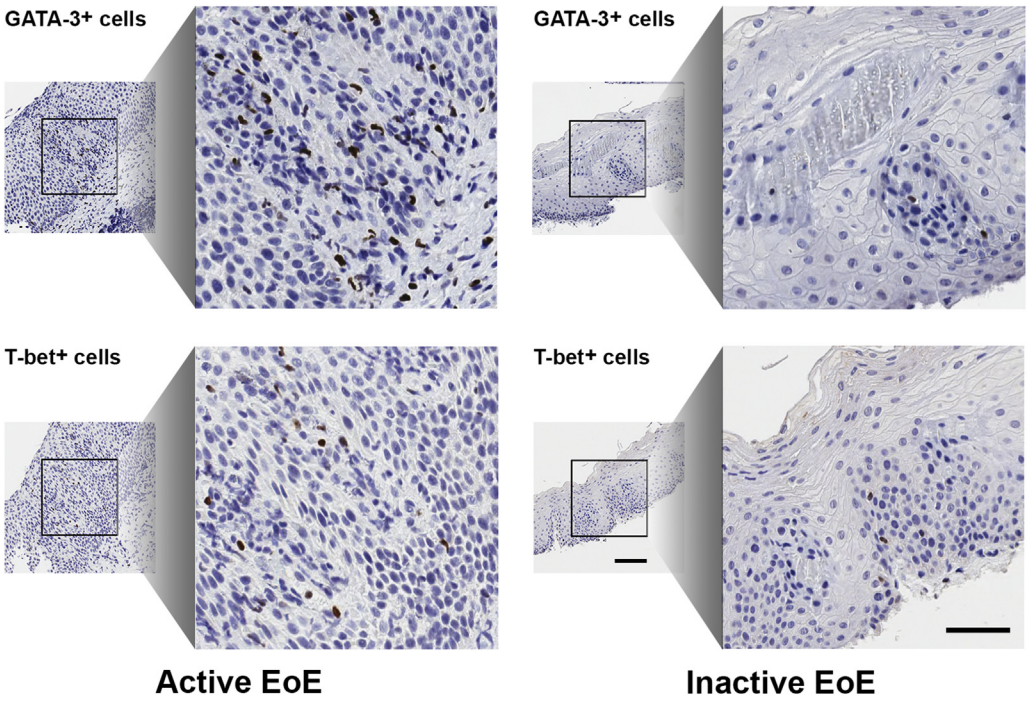

FIG 1. Subjects with EoE have increased GATA-3 and T-bet expression. Immunohistochemistry stains for GATA-3 and T-bet in the same subject with active and inactive EoE.

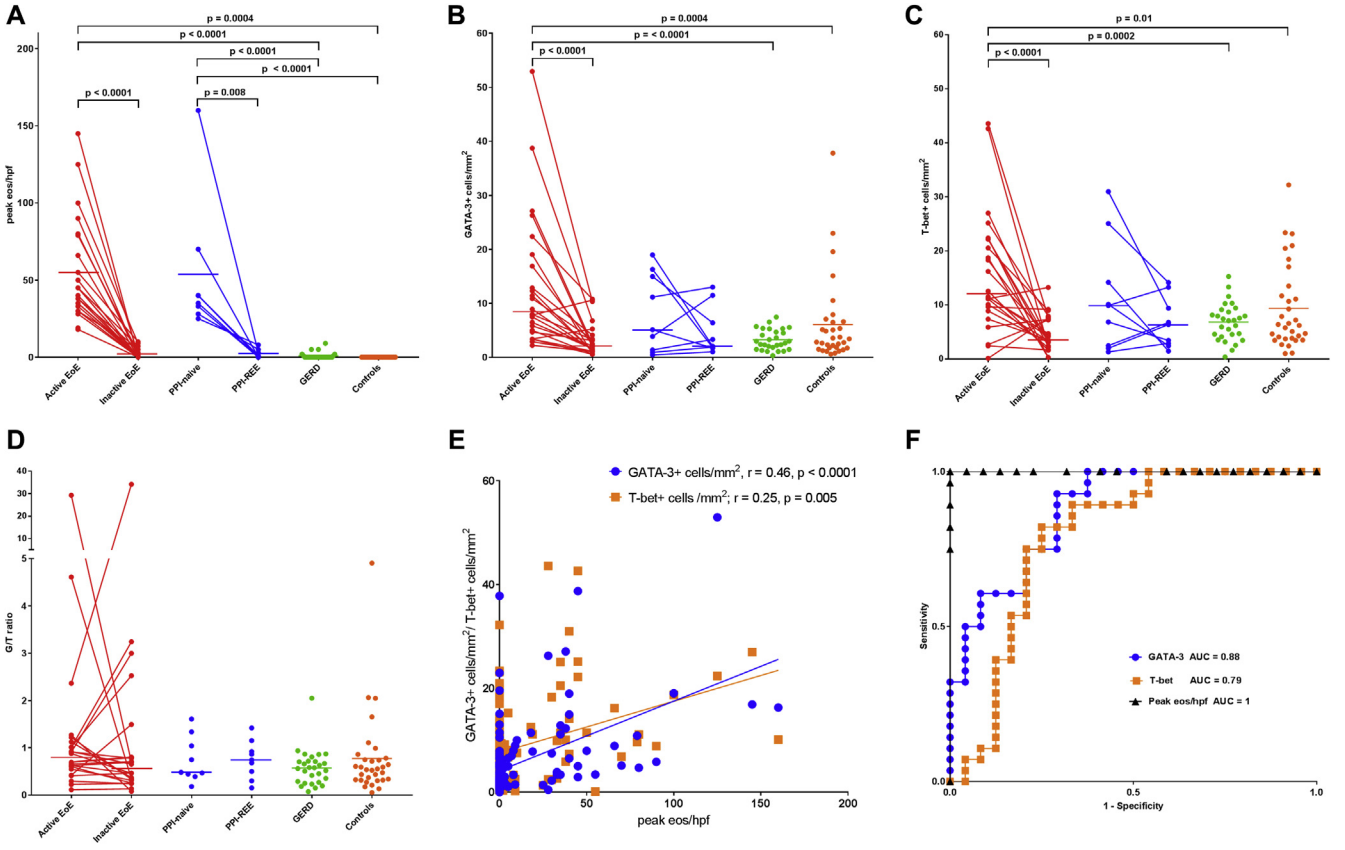

FIG 2. GATA-3+ cells and T-bet+ cells are increased in subjects with EoE, correlate with esophageal eosinophilia, and decrease in response to treatment. Peak esophageal eosinophil counts are shown in **A**. Expression of GATA-3 **(B)** and T-bet **(C)** is increased in EoE relative to GERD and health controls. Similar trends were noted for subjects with PPI-REE but these did not reach statistical significance. The G/T ratio does not distinguish subjects with EoE or PPI-REE **(D)**. GATA-3 and T-bet correlate with eos/hpf **(E)**. Receiver-operating characteristic curves for eos/hpf, GATA-3+ cells/mm^2, and T-bet+ cells/mm^2 suggest that GATA-3 and T-bet expressions may serve as histologic biomarkers that differentiate active EoE from GERD **(F)**.

EoE ($P < .0001$) (see Fig 2, *B* and *C*). A similar trend was observed for subjects with PPI-REE, such that those with inactive EoE/PPI-REE were indistinguishable from those with GERD and control subjects based on nuclear transcription factor expression. No significant differences were observed between groups for the G/T ratio (see Fig 2, *D*).

While EoE has been defined as a type 2 cytokine-mediated disease, we observed similar increases in GATA-3+ and T-bet+ cells in subjects with active EoE and PPI-REE. Expression of both markers was associated with active disease and the G/T cellular ratio did not serve as a histologic marker of EoE or PPI-REE. The observed differences were almost identical when the relative percentages of GATA-3+ and T-bet+ cells were compared among groups (see Fig E2 in this article's Online Repository at www.jacionline.org), suggesting tissue polarization in addition to cellular infiltration. Our observations corroborate earlier findings[5] suggesting that EoE and PPI-REE share a common pathophysiology and that PPIs, topical corticosteroids, and dietary elimination each result in decreased GATA-3 expression. The significance of increased T-bet expression is unclear but suggests that a mixed T_H2/T_H1 inflammatory infiltrate is associated with active disease. Consistent with this finding, others have demonstrated increased expression of T_H1 cytokines (IFN-γ[7] and TNF-α[8]) in EoE.

We acknowledge the limitation that this is a retrospective, single-center study of pediatric subjects; therefore, the results cannot be generalized to adults. Second, immunohistochemical assessments were performed on only a single biopsy specimen from each endoscopy; therefore, it is possible that the immune polarization described is confined to areas of maximum eosinophil infiltrate. Although GATA-3 and T-bet expression was similar in EoE and PPI-REE, we did not examine a sufficient number of subjects with PPI-REE in order to observe statistical differences between groups or following treatment for subjects with PPI-REE. Finally, we did not perform dual staining to ensure that the cells positive for GATA-3 and T-bet were CD4+ helper T cells; therefore, these transcription factors may be expressed by other cell types, including innate lymphoid cells, regulatory T cells,[9] and natural killer cells (see Fig E3 in this article's Online Repository at www.jacionline.org). However, these limitations are balanced by a number of strengths including detailed clinical information, automated assessment of GATA-3/T-bet expression, and blinded analysis of tissue samples.

In summary, we observed that subjects with EoE have increased GATA-3 and T-bet expression when compared with subjects with GERD and healthy controls. Similar trends were noted for subjects with PPI-REE. Automated assessment of the GATA-3 and T-bet expression may be a useful strategy to distinguish subjects with EoE/PPI-REE and GERD; moreover, these markers may also be useful in assessing treatment response.

Benjamin L. Wright, MD[a,b]
Nathalie Nguyen, MD[c,d]
Kelly P. Shim, BS[a,b]
Joanne C. Masterson, PhD[c,d]
Elizabeth A. Jacobsen, PhD[a]
Sergei I. Ochkur, PhD[a]
James J. Lee, PhD[a]
Glenn T. Furuta, MD[c,d]

From [a]Mayo Clinic Arizona, Scottsdale, Ariz; [b]Phoenix Children's Hospital, Phoenix, Ariz; [c]the Digestive Health Institute, Children's Hospital of Colorado, Aurora, Colo; and [d]the Gastrointestinal Eosinophilic Diseases Program, University of Colorado School of Medicine, Aurora, Colo. E-mail: wright.benjamin@mayo.edu.

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High number of early respiratory infections in association with allergic sensitization to mold promotes childhood asthma

To the Editor:

The relationships among early life respiratory infections, aeroallergen sensitization, and subsequent development of childhood asthma is unclear. To examine the effects of respiratory infections and allergic sensitization on asthma development, data from the Cincinnati Childhood Allergy and Air Pollution Study (CCAAPS) birth cohort were evaluated.

METHODS

Study Population

Pediatric subjects (aged 1-18 years) were retrospectively identified at the Children's Hospital of Colorado from 2006 to 2016. This study was approved by the Colorado Multiple Institutional Review Board (institutional review board approval no. 07-0888).

Case definitions, clinical data, and biospecimen collection

Diagnosis of active EoE was defined by consensus guidelines.[E1] Subjects with PPI-REE were treated with high-dose PPI for at least 8 weeks and had less than 15 eos/hpf on repeat endoscopic biopsy. Subjects with GERD displayed symptoms consistent with reflux as defined by a pediatric gastroenterologist or had an abnormal pH/impedance study. Control subjects had gastrointestinal symptoms necessitating an upper endoscopy with normal histology (0 eos/hpf). Clinical data were collected through retrospective chart review. Asthma, eczema, seasonal allergies, and IgE-mediated food allergies were determined by clinical history as documented in the medical record. Tissue analysis was performed using paraffin-embedded esophageal tissue sections from the Children's Hospital of Colorado. Esophageal eosinophil counts were quantified by a pediatric pathologist. Study personnel were blinded as to case/control status during sample analysis.

Immunohistochemical staining of GATA-3 and T-bet

Tissue sectioning and immunohistochemical staining were performed at the Pathology Research Core (Mayo Clinic, Rochester, Minn) using the Leica Bond RX stainer (Leica Biosystems, Nussloch, Germany). Five-micron formalin-fixed, paraffin-embedded (FFPE) tissue sections were treated with Bond Epitope Retrieval 1 (Citrate; Leica Biosystems, Newcastle, UK) for 20 minutes and Protein Block Serum-Free (Dako, Carpinteria, Calif) for 5 minutes. GATA-3 (clone L50-823, Biocare Medical, Pacheco, Calif) and T-bet (SC-21003, Santa Cruz Biotech, Dallas, Tex) were diluted in Antibody Diluent, Background Reducing (Dako, Carpinteria, Calif) at 1:400 and 1:150, respectively, and applied to tissue for 15 minutes.

The detection system used was Bond Polymer Refine Detection System (Leica Biosystems). This system includes the hydrogen peroxidase block, postprimary and polymer reagent, 3′-diaminobenzidine (DAB), and hematoxylin. Immunostaining visualization was achieved by incubating the slides for 10 minutes in DAB and DAB buffer (1:19 mixture) from the Bond Polymer Refine Detection System. To this point, slides were rinsed between steps with 1× Bond Wash Buffer (Leica Biosystems). Slides were counterstained for 5 minutes using Schmidt hematoxylin and molecular biology grade water (1:1 mixture), followed by several rinses in 1× Bond wash buffer and distilled water; this is not the hematoxylin provided with the Refine kit. Once the immunohistochemistry process was completed, slides were removed from the stainer and rinsed in tap water for 5 minutes. Slides were dehydrated in increasing concentrations of ethyl alcohol and cleared in 3 changes of xylene before permanent coverslipping in xylene-based medium.

Image analysis

Tissue sections were digitized (Aperio AT Turbo; Leica Biosystems, Buffalo Grove, Ill) and analyzed using Aperio ImageScope software (version 11.2.0.780; Aperio Technologies, Vista, Calif). Serial sections of esophageal biopsies where the maxiumum eosinophil focus was located were analyzed for each subject. The submucosal layer was outlined to subtract it from analysis (see Fig E1). Image analysis using a nuclear algorithm was used to quantify the cells staining positive for T-bet and GATA-3. Only those nuclei that stained strongly or moderately positive according to the algorithm were considered positive.

Statistical analysis

Clinical characteristics of the cases and controls were summarized with descriptive statistics. Baseline comparisons of median eos/hpf, GATA-3+ cells/mm^2, T-bet+ cells/mm^2, and G/T ratios were made with a Mann-Whitney test. Paired comparisons of G/T ratios pretreatment and posttreatment (subjects with EoE and PPI-REE) were performed using a Wilcoxon matched-pairs signed-rank test. Spearman rho values were used to assess correlations between eos/hpf and GATA-3+ cells/mm^2 or T-bet+ cells/mm^2. Statistical comparisons and plots were made with GraphPad Prism (version 7.00 for Windows; GraphPad software, San Diego, Calif).

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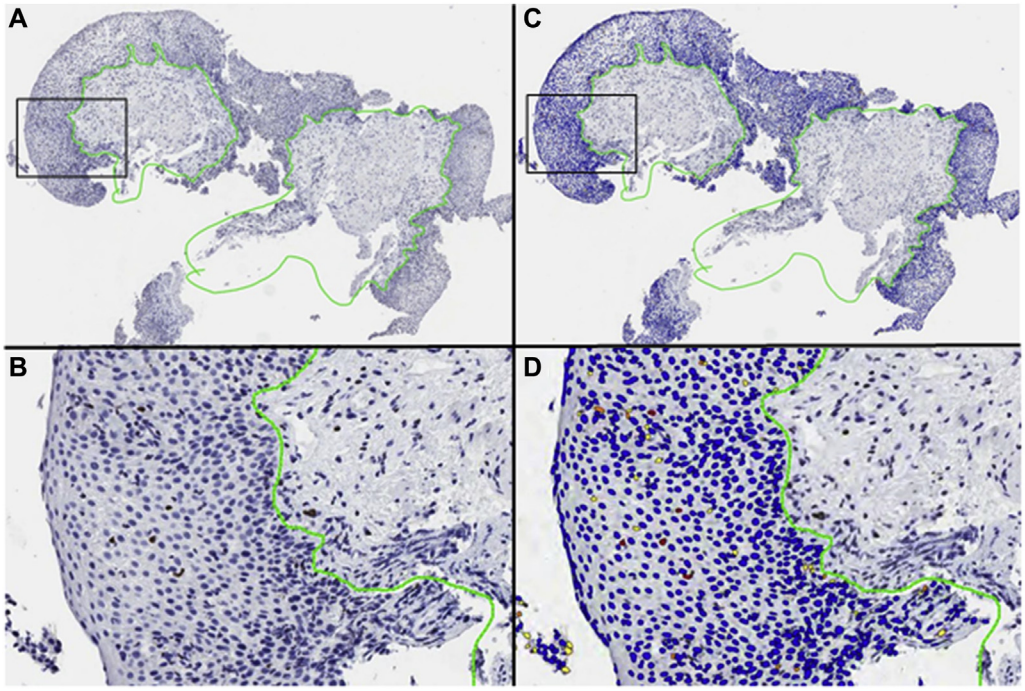

FIG E1. Image analysis of GATA-3/T-bet expression in the esophageal epithelium is an automated assessment of immune polarization. The submucosal layer was outlined to exclude it from analysis. DAB stains for GATA-3 of the esophageal epithelium are shown at low power **(A)** and high power **(B)**. Image analysis software using a nuclear algorithm quantifies the cells staining strongly positive (red), moderately positive (orange), and weakly positive (yellow) for GATA-3. Corresponding images are shown in **C** and **D**. The number of cells was divided by the epithelial area (mm^2) analyzed. Cells staining weakly positive were not considered positive in the data analysis.

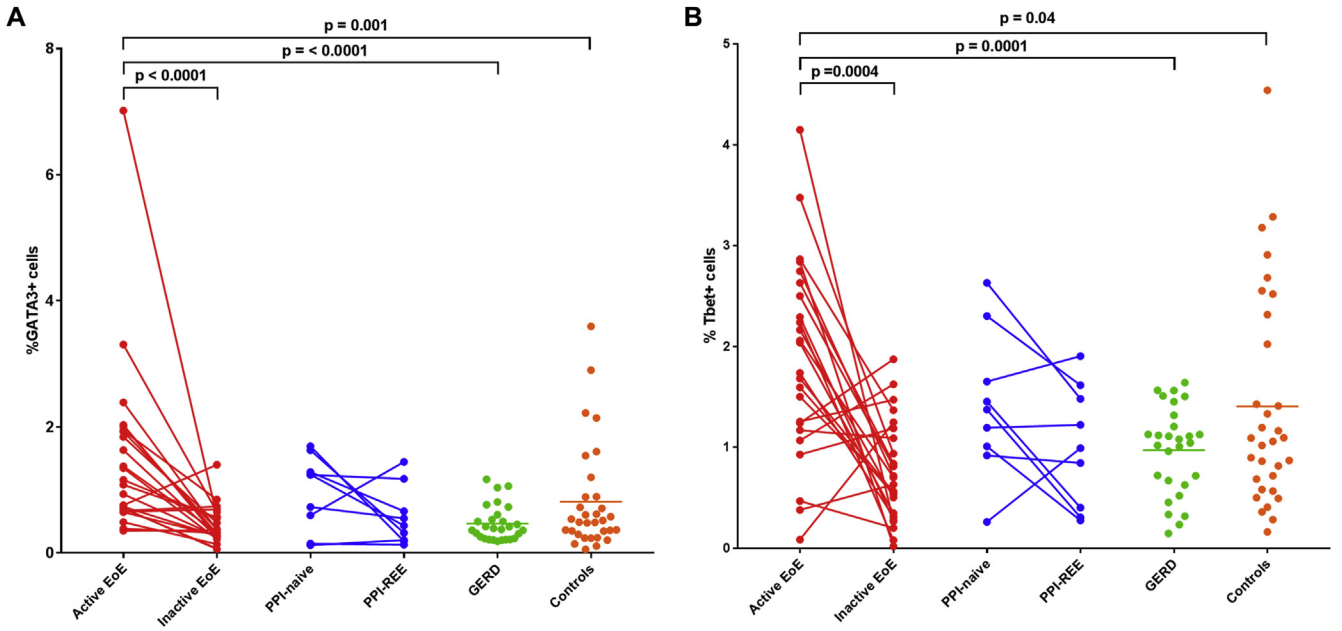

FIG E2. %GATA+ **(A)** and %T-bet+ **(B)** cells are increased in active EoE relative to GERD and controls and decrease in response to treatment similar to the number of GATA-3+ and T-bet+ cells when analyzed per unit area (mm^2). GATA-3+ and T-bet+ cells were quantified as a percentage of the total number of nucleated cells present.

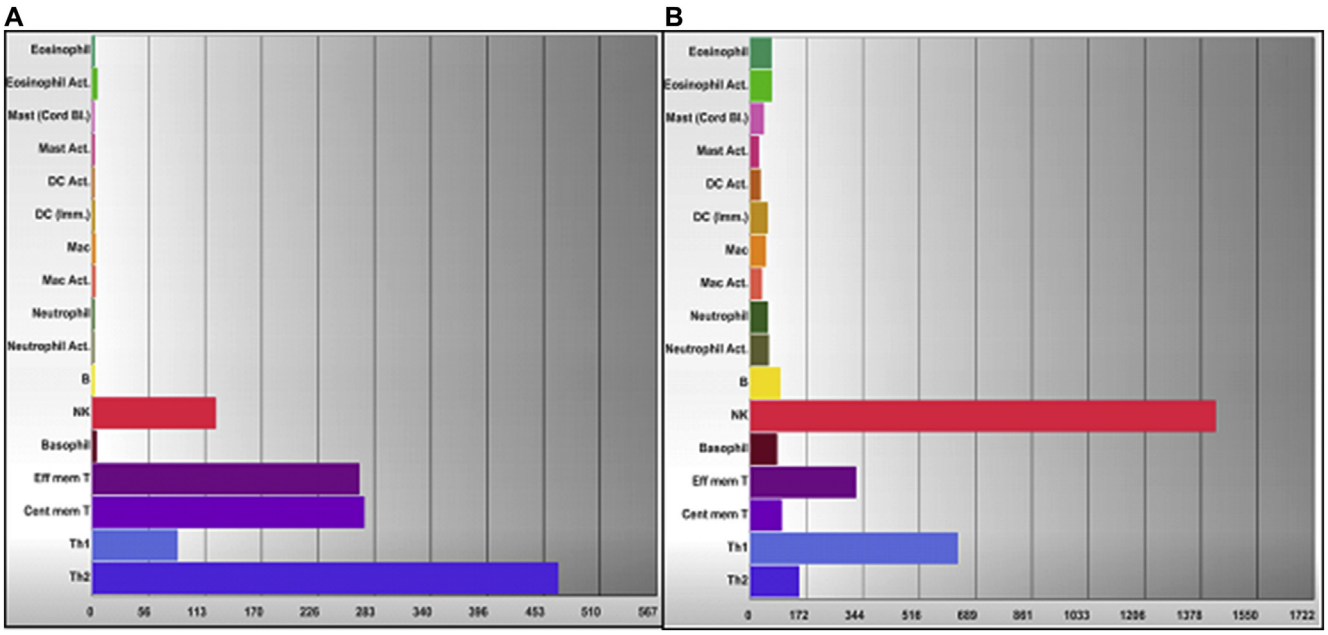

FIG E3. GATA-3 (probe set 209604_s_at, panel **A**) and TBX21 (encoding T-bet, probe set 220684_at, panel **B**) expression patterns in human immune cells. Data publicly accessible from the Immunological Genome Database at http://www.immgen.org/databrowser. *DC*, Dendritic cell; *NK*, natural killer.

TABLE E1. Baseline characteristics of the study population

Characteristic	GERD (n = 28)	PPI-REE (n = 9)	EoE cases (n = 24)	Controls (n = 32)
Age (y), mean ± SD	3.7 ± 2.1	7.6 ± 5.5	4.0 ± 2.8	12.0 ± 4.9
Sex: male, n (%)	21 (75)	8 (89)	22 (92)	13 (41)
White, n (%)	23 (82)	8 (89)	23 (96)	19 (59)
Atopic conditions, n (%)				
Asthma	13 (46)	4 (44)	10 (42)	0 (0)
Eczema	4 (14)	2 (22)	11 (46)	2 (6)
Seasonal allergies	12 (43)	2 (22)	11 (46)	1 (3)
Food allergies	4 (14)	3 (33)	14 (58)	1 (3)
Peak eosinophil counts, mean eos/hpf ± SD				
Baseline	0.9 ± 2.1	53.9 ± 45.0	54 ± 33.2	0
Posttreatment	—	1.9 ± 2.9	2.2 ± 3.3	—