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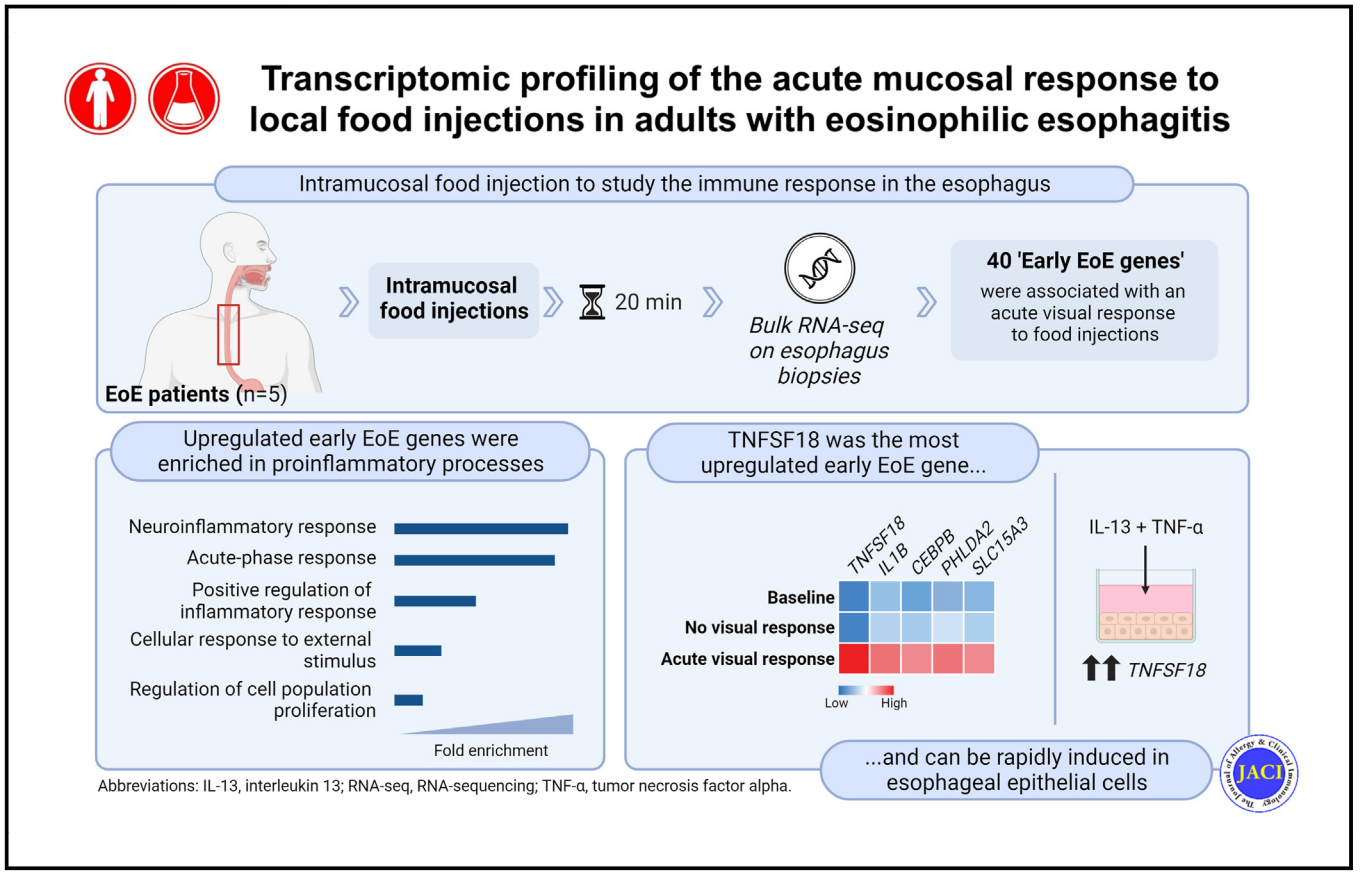
GRAPHICAL ABSTRACT



Capsule summary: This study analyzed early changes in the esophageal transcriptome triggered by intramucosal food injections in patients with EoE, providing insight into genes and functional processes involved in the acute mucosal response to food in EoE.

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Transcriptomic profiling of the acute mucosal response to local food injections in adults with eosinophilic esophagitis

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Background: Exposure of the esophageal mucosa to food allergens can cause acute mucosal responses in patients with eosinophilic esophagitis (EoE), but the underlying local immune mechanisms driving these acute responses are not well understood.
Objective: We sought to gain insight into the early transcriptomic changes that occur during an acute mucosal response to food allergens in EoE.
Methods: Bulk RNA sequencing was performed on esophageal biopsy specimens from adult patients with EoE (n = 5) collected before and 20 minutes after intramucosal injection of various food extracts in the esophagus. Baseline biopsy specimens from control subjects without EoE (n = 5) were also included.
Results: At baseline, the transcriptome of the patients with EoE showed increased expression of genes related to an EoE signature. After local food injection, we identified 40 genes with a potential role in the early immune response to food allergens (most notably *CEBPB, IL1B, TNFSF18, PHLDA2,* and *SLC15A3*). These 40 genes were enriched in processes related to immune activation, such as the acute-phase response, cellular responses to external stimuli, and cell population proliferation. *TNFSF18* (also called *GITRL*), a member of the TNF superfamily that is best studied for its costimulatory effect on T cells, was the most dysregulated early EoE gene, showing a 12-fold increase compared with baseline and an 18-fold increase compared with a negative visual response. Further experiments showed that the esophageal epithelium may be an important source of *TNFSF18* in EoE, which was rapidly induced by costimulating esophageal epithelial cells with the EoE-relevant cytokines IL-13 and TNF-α.
Conclusions: Our data provide unprecedented insight into the transcriptomic changes that mediate the acute mucosal immune response to food allergens in EoE and suggest that *TNFSF18* may be an important effector molecule in this response. (J Allergy Clin Immunol 2024;153:780-92.)

Abbreviations used
CPM: Count per million
Ct: Cycle threshold
DEG: Differentially expressed gene
EoE: Eosinophilic esophagitis
FC: Fold change
FDR: False discovery rate
GO: Gene Ontology
ILC2: Group 2 innate lymphoid cell
KEGG: Kyoto Encyclopedia of Genes and Genomes
RNA-seq: RNA sequencing

Key words: *Acute response, eosinophilic esophagitis, esophagus, food challenge,* GITRL, *RNA sequencing,* TNFSF18

Eosinophilic esophagitis (EoE) is a chronic, allergen-driven disorder of the esophagus characterized by the infiltration of eosinophils in the esophageal mucosa and symptoms related to esophageal dysfunction.[1] The prevalence of EoE is approximately 1 in 3000, with a male-to-female ratio of 3:1.[2] Food allergens play an important role in the pathogenesis of EoE, as demonstrated by endoscopic and clinical resolution of EoE once the causative food is removed from the diet and exacerbation when the same food is reintroduced.[3] Similarly, amino acid–based elemental diets are effective in both adults and children with EoE.[4-8] Type 2 inflammation represents an important subset of the relevant immune pathways activated during EoE. This is supported by studies that show local expression of cytokines such as IL-4, IL-5, and IL-13[9]; increased numbers of esophageal T_H2 cells, mast cells, eosinophils, basophils, B cells, and group 2 innate lymphoid cells (ILC2s)[10-14]; and an association of EoE with other atopic disorders.[15,16] However, the exact mechanism by which food allergens can initiate inflammation in EoE is still unknown, as there are limited data on the early local esophageal immune response after challenge with a specific food trigger.

Previous studies have provided insight into transcriptional changes associated with active EoE.[17-19] The EoE transcriptome is enriched in genes functionally involved in eosinophilia, immunity, and atopy.[19] The IL-13–induced gene *CCL26*, which encodes eotaxin-3, is the most upregulated gene in patients with EoE compared with control subjects (279-fold) and strongly correlates with disease severity.[17] Other highly induced genes include *POSTN, CAPN14, LRRC31,* and *ANO1*.[17,20-22] Downregulated genes in EoE are related to epithelial homeostasis,[17] such as *DSG1*.[23] Furthermore, long noncoding

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RNAs, a type of RNA that are not translated into protein, have been shown to play a role in EoE pathophysiology and may help in diagnosis and monitoring disease activity.[19]

However, little emphasis has been placed on characterizing genes that mediate the acute esophageal immune response to food allergens. Recently, we injected food extracts into the esophageal mucosa of adult patients with EoE during upper endoscopy and monitored acute mucosal responses for 20 minutes.[24] These food injections induced acute responses of the esophageal mucosa, such as edema, erythema, and smooth muscle contraction, in various degrees of severity in patients with EoE. The fact that these food-induced acute esophageal responses could be responsible for painful esophageal symptoms and potentially exacerbate esophageal inflammation stresses the need for better understanding of the cellular and molecular processes mediating such reactions. This was also stressed in a recent article describing these symptoms as food-induced immediate response of the esophagus.[25] Therefore, in this study, we aimed to gain insight into the early transcriptomic changes that occur during an acute mucosal response to food allergens in EoE. For this purpose, we performed bulk RNA sequencing (RNA-seq) on esophageal biopsy specimens collected before and 20 minutes after intramucosal food injections in the esophagus of adult patients with EoE.

METHODS

Study subjects, design, and sample collection

Detailed methods regarding study subjects and design were previously described.[24] Briefly, adult (age 18-75 years) patients with EoE were included from the outpatient clinic of the Amsterdam UMC between August 2019 and 2021. Adults were eligible for enrollment if EoE was previously diagnosed according to current guidelines, defined as the presence of >15 eosinophils per high-power field and typical symptoms of EoE (eg, dysphagia and food impaction). Exclusion criteria were 1) inability to stop topical corticosteroids, β-blockers, or angiotensin-converting enzyme inhibitors; 2) use of oral or systemic antihistaminics, oral cromoglicates, systemic corticosteroids, leukotriene inhibitors, or monoclonal antibodies in the month preceding the study; 3) proven gastroesophageal reflux disease or other cause for esophageal eosinophilia; 4) history of peptic ulcer disease, Barrett esophagus, or gastrointestinal cancer; 5) severe comorbidity as indicated by American Society of Anesthesiologists class III, VI, or V; and 6) history of anaphylaxis or a severe systemic reaction to previous allergy tests (grade 3 or 4). Adults who underwent endoscopy for reasons other than esophageal symptoms were included as control subjects. Signed informed consent to participate in the study was obtained from patients and control subjects.

All consented patients presented with the typical symptoms and endoscopic signs of EoE at the time of endoscopy and were not allowed to use immunosuppressive drugs during the trial. During endoscopy, the esophageal mucosa of patients with EoE was locally injected with 6 different foods and a negative control (0.9% NaCl). Three foods were selected based on the most prevalent sensitizations in EoE (cow's milk, wheat, and apple),[26] and another 3 foods were included based on the patient's history of clinically suspected foods. The injections were done in a prespecified order at 3-cm intervals in axial length, alternating at the 3- and 9-o'clock and 6- and 12-o'clock positions. Acute local visual responses were monitored by endoscopy for up to 20 minutes.[24] Baseline biopsy specimens were collected before the injections and 20 minutes after the injections; specimens were collected from each of the 7 injection sites. These biopsy specimens and baseline biopsy specimens from 5 control subjects without EoE were collected in RNAlater (Qiagen, Hilden, Germany) and stored at −80°C until further use.

Biopsy specimens from 5 patients with EoE were used for RNA-seq. From each of the 5 patients, we included one baseline biopsy specimen, one specimen from a positive visual response to food injection, and one specimen from a negative visual response to food injection, totaling 15 specimens (3 biopsy specimens × 5 patients). In this way, each patient served as their own control. If a patient had positive visual responses to multiple injections with different food extracts, the most severe response was used for the analysis. Biopsy specimens from a negative visual response to injections were obtained at the greatest distance from sites with a positive visual response to prevent possible interference.

Sample library preparation, RNA-seq, and data analysis

Esophageal biopsy specimens stored in RNAlater at −80°C were homogenized in 600 μL Buffer RLT (Qiagen) plus 1% β-mercaptoethanol using the Precellys homogenizer (VWR International, Graumanngasse, Vienna). Homogenates were centrifuged (2 minutes; 14,000 rpm; room temperature), and DNA, RNA, and protein were extracted using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen) per manufacturer's instructions. DNA, RNA, and protein were stored at −80°C until further use.

Sample quality control measures were provided by Novogene (Beijing, China), and libraries were constructed from samples of acceptable quality using the Novogene NGS RNA Library Prep Set (PT042). Library quantification was performed using Qubit 2.0 (Thermo Fisher Scientific, Waltham, Mass) and real-time PCR, and size distribution selection was performed using the Bioanalyzer (Agilent, Santa Clara, Calif) system. Quantified libraries were sequenced on Illumina NovaSeq 6000 (Illumina, San Diego, Calif) (sequencing strategy PE150) at the Novogene sequencing laboratory in Cambridge, United Kingdom, and paired-end reads were generated. For quality control of the raw data, raw reads of FASTQ format were first processed through in-house Perl scripts to obtain clean reads. Reads containing adapter, reads containing ploy-N, and low-quality reads were removed from the raw data. In addition, Q20, Q30, and GC content of the clean data were calculated. All downstream analyses were based on the high-quality clean data. Paired-end clean reads were aligned against the GRCh38 human reference genome using HISAT2 v2.0.5.[27] FeatureCounts v1.5.0-p3[28] was used to generate read counts mapped to each gene. Read counts data were analyzed using iDEP v0.96.[29] The expression threshold for downstream analysis was set at a minimum of 1 count per million (CPM) in at least 2 samples to remove low-abundance genes, and counts data were transformed using edgeR[30]: log2(CPM + c), where constant (c) = 4. Differentially expressed genes (DEGs) were identified by DESeq2.[31] Fold changes (FCs) were assessed, and *P* values were corrected for multiple testing using false discovery rate (FDR), generating adjusted *P* values. Genes were considered differentially expressed if FC was > 1.5 and FDR was < 0.05. For the analysis of food injections, DESeq2

TABLE I. Patient characteristics

Patient ID	Sex	Age (y)	PEC	Positive visual response	Negative visual response
1	M	26	45	**Tomato** (moderate narrowing/edema)	**Milk**
3	M	48	5	Peanut (moderate edema/rings)	**Chicken**
4	M	52	100	**Wheat** (moderate edema)	**Beer**
8	M	26	30	**Mango** (moderate edema)	**Grape**
10	F	22	50	Apple (moderate edema)	Soy

Foods presented in bold were suspected by the patient to cause symptoms.
F, Female; *M,* male; *PEC,* peak eosinophil count (at time of endoscopy).

ran paired tests by using the following statistical model: Gene expression ~ response + patient ID, where response (baseline, negative, positive) is the factor variable, and patient ID is the fixed factor to pair samples.

Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of specific gene clusters were performed using ShinyGO v0.76.1 with the full set of expressed genes as background.[32] Enriched GO terms or KEGG pathways were considered significant if FDR was < 0.05.

Cell culture

The immortalized human esophageal epithelial cell line EPC2-hTERT[33-35] (provided by Dr. Anil Rustgi, University of Pennsylvania, Philadelphia, Pa) was cultured in low-calcium ($[Ca^{2+}]$ = 0.09 mM) Keratinocyte SFM (Thermo Fisher Scientific; cat. 10725-018) supplemented with bovine pituitary extract (50 μg/mL) (Thermo Fisher Scientific; cat. 13028-014), epidermal growth factor (1 ng/mL) (Thermo Fisher Scientific; cat. 10450-013), and penicillin (100 U/mL)/streptomycin (100 μg/mL) (Thermo Fisher Scientific; cat. 15140-122). Cultures were tested every month for mycoplasma contamination.

For stimulation experiments, EPC2-hTERT cells were grown to confluence in low-calcium Keratinocyte SFM in a 48-well cell culture plate (Corning Inc, Corning, NY; cat. 3548). Initial differentiation of confluent EPC2-hTERT monolayers was induced by switching to high-calcium Keratinocyte SFM ($[Ca^{2+}]$ = 1.8 mM) for 5 days. EPC2-hTERT cells were then stimulated with IL-13 (100 ng/mL) (Prospec-Tany TechnoGene Ltd, Rehovot, Israel; cat. CYT-446), TNF-α (100 ng/mL) (Thermo Fisher Scientific; cat. PHC3016), and IL-13 + TNF-α (both 100 ng/mL). At 20 minutes, 1 hour, and 4 hours after stimulation, EPC2-hTERT cultures were lysed in 350 μL Buffer RLT plus 1% β-mercaptoethanol for total RNA isolation with the RNeasy Mini Kit (Qiagen) per manufacturer's instructions.

RT-PCR

Total RNA was subjected to reverse transcription using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, Calif). RT-PCR was performed on a CFX Opus 384 Real-Time PCR System (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). PrimePCR SYBR Green assays for *CEBPB* (unique Assay ID: qHsaCED0019041), *IL1B* (qHsaCID0022272), *PHLDA2* (qHsaCED0047473), *SLC15A3* (qHsaCED0001796), and *TNFSF18* (qHsaCED 0043856) were purchased from BioRad. Results were normalized to ribosomal protein S13 (*RPS13*; qHsaCID0038672). mRNA expression levels were calculated by subtracting *RPS13* cycle threshold (Ct) from the gene of interest Ct to obtain ΔCt. For gene expression analysis in biopsy specimens, the relative mRNA expression was calculated using the following formula: mRNA expression = 100,000 × $(2^{-\Delta Ct})$. For gene expression analysis in EPC2-hTERT *in vitro* experiments, the medium control ΔCt was subtracted from the stimulated condition ΔCt to obtain ΔΔCt. mRNA expression was calculated using the following formula: FC = $2^{-\Delta\Delta Ct}$.

Statistical analysis

RNA-seq data were analyzed using iDEP v0.96 as described above. Further statistical analyses were performed using GraphPad Prism v9.4.1 (GraphPad Software, Boston, Mass). Statistical significance was determined by unpaired t test (normal distribution, equal variance, 2 groups), Welch t test (normal distribution, unequal variance, 2 groups), or (repeated measures) one-way ANOVA followed by Bonferroni multiple comparisons test (normal distribution, equal variance, ≥3 groups). *P* values of <.05 were considered significant.

Study approval

This study was approved by the Medical Ethics Committee of the Amsterdam UMC. All subjects provided written informed consent (https://trialsearch.who.int/, ID: NL7781).

Data availability

Bulk RNA-seq data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (accession number GSE246323).

RESULTS

Transcriptomic characteristics of the EoE patient cohort

First, we aimed to determine the baseline inflammatory status of the esophagus of the 5 patients with EoE included in this study and how it compares to previous reports. Patient characteristics are provided in Table I. We subjected biopsy specimens collected before the food injections from each of the 5 patients and baseline biopsy specimens from 5 control subjects without EoE to bulk RNA-seq. A total of 15,203 genes passed the expression threshold of at least 1 CPM in 2 samples. Among these expressed genes, 323 genes (2.12%) were dysregulated (FC > 1.5, FDR < 0.05) and showed high similarity in transcript expression patterns among patients with EoE. Of the 323 dysregulated genes, 211 genes (65.3%) were upregulated and 112 genes (34.7%) were downregulated in patients with EoE compared with control subjects (Fig 1, *A*). Similar to previous EoE transcriptome

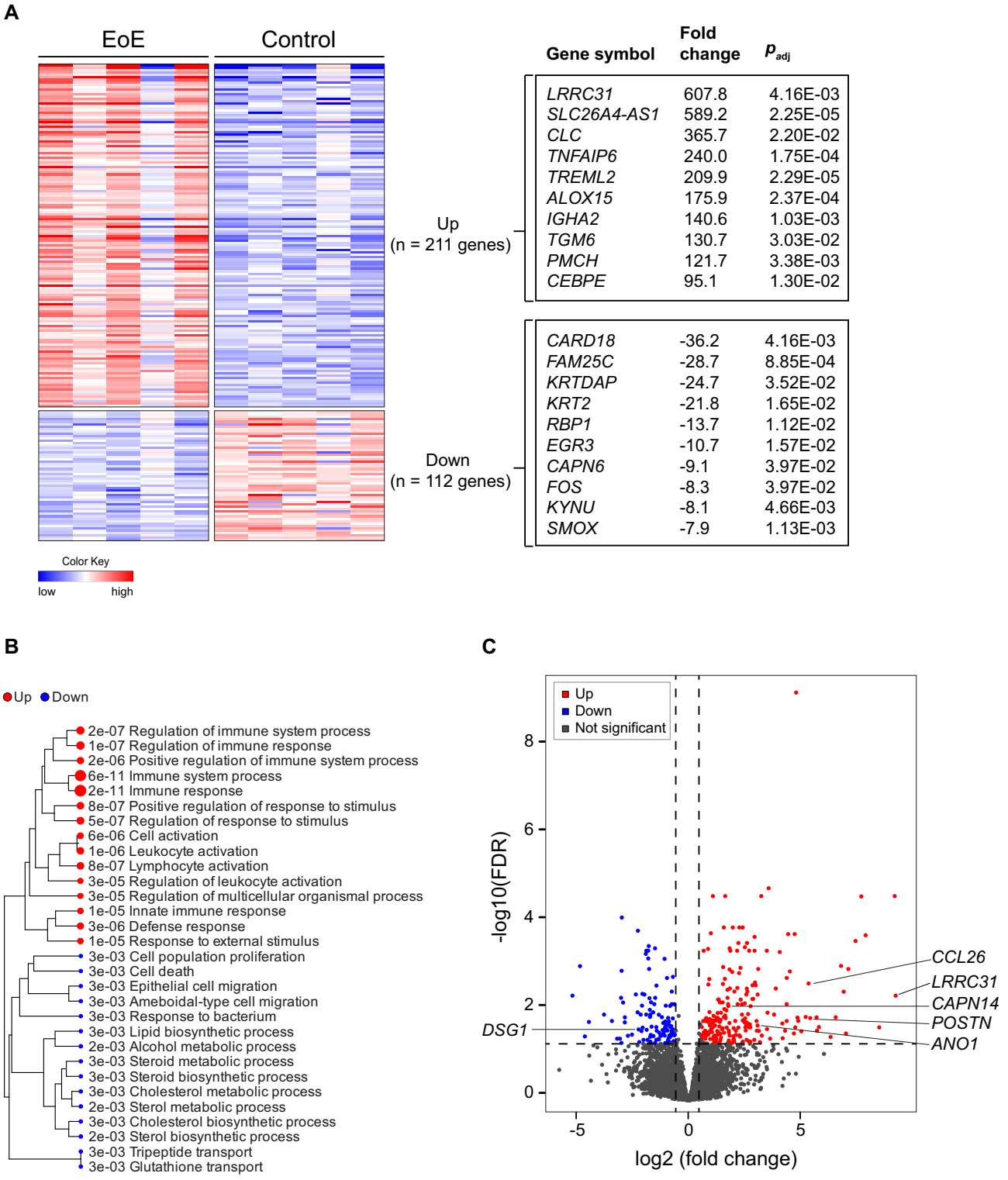

FIG 1. Gene expression analysis by RNA-seq in esophageal biopsy specimens from patients with active EoE and control subjects without EoE. **(A)** Heatmap of the 323 genes that were identified as dysregulated (FC > 1.5, FDR < 0.05) in EoE patients (n = 5) compared with control subjects (n = 5). The 10 most dysregulated genes in each cluster along with their FC (EoE vs control) and adjusted *P* value are indicated on the right. Each column represents an individual patient or control subject, and each row represents a gene. **(B)** Hierarchical clustering tree of enriched biological processes that are upregulated *(red)* or downregulated *(blue)* in EoE patients vs control subjects, with dot size inversely corresponding to the adjusted *P* value. **(C)** Volcano plot showing log2 FC values by −log10 FDR values for all 15,203 expressed genes. Significantly upregulated genes (n = 211 genes) are *red*, significantly downregulated genes (n = 112 genes) are *blue*, and nonsignificant genes are *gray*. *Dashed lines* represent the thresholds used for FDR (< 0.05) and FC (> 1.5). Genes that were previously identified as part of the EoE transcriptome are indicated.

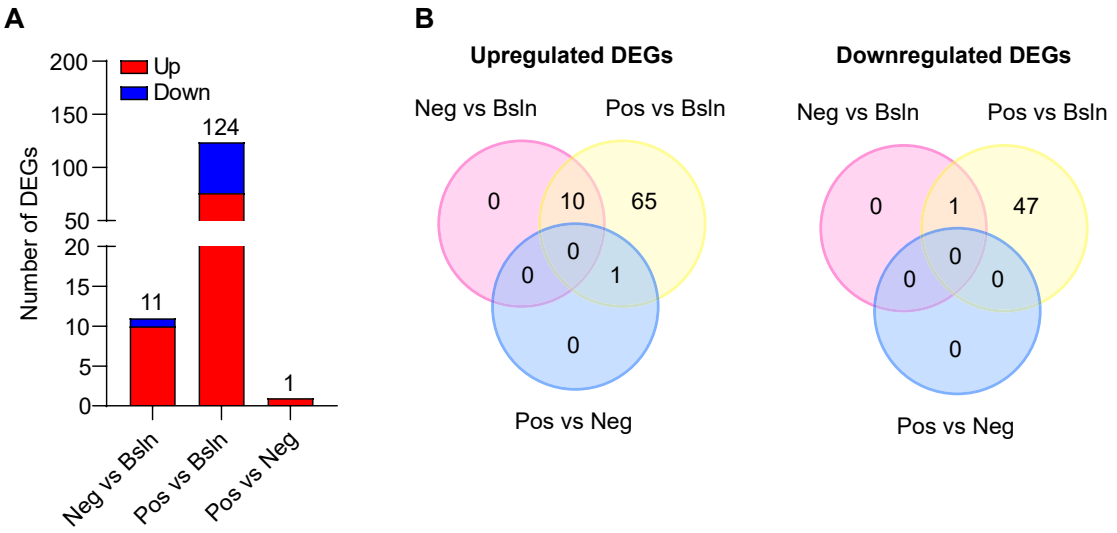

FIG 2. Gene expression analysis by RNA-seq in esophageal biopsy specimens from patients with active EoE 20 minutes after local challenge by intramucosal food injections. **(A)** Number of significantly upregulated and downregulated DEGs (FC > 1.5, FDR < 0.05) in 3 different comparison groups: negative visual response vs baseline (Neg vs Bsln), positive visual response vs baseline (Pos vs Bsln) and positive visual response vs negative visual response (Pos vs Neg). **(B)** Venn diagrams depicting significantly upregulated *(left)* and downregulated DEGs *(right)* that are unique to or shared by the different comparison groups.

studies,[17,19,20,22] we found robust upregulation of *LRRC31* (608-fold, $P = .004$), *POSTN* (43-fold, $P = .014$), *CCL26* (41-fold, $P = .002$), *ANO1* (8.5-fold, $P = .020$), and *CAPN14* (3.4-fold, $P = .006$) and downregulation of *DSG1* (5-fold, $P = .024$) in patients with EoE compared with control subjects (Fig 1, *C*). All 323 DEGs along with their FC and adjusted *P* value are provided in Table E1 (in the Online Repository at www.jacionline.org).

Consistent with the distinct transcriptional signatures, GO enrichment analysis showed that the overexpressed genes were primarily involved in immune cell activation and (regulation of) the immune response. The downregulated genes related to a variety of functional/homeostatic processes (Fig 1, *B*). Altogether, the transcriptome of our EoE patient cohort shows interindividual similarities and compares with previously published EoE transcriptome studies, setting a solid basis for further analyses.

Identifying genes associated with acute responses of the esophageal mucosa to food injections

The primary aim of this study was to characterize gene expression signatures and functional processes of the acute mucosal response to food injections. To do this, we subjected esophageal biopsy specimens collected before (baseline) and 20 minutes after a negative and after a positive visual response to esophageal challenge by intramucosal food injections to bulk RNA-seq. The positive visual responses included in the analysis were not induced by the same food extracts in each patient (Table I). We used 15 biopsy specimens for gene expression analysis (3 biopsy specimens × 5 patients). A total of 15,417 genes passed the expression filter of CPM ≥ 1 in at least 2 samples. Samples were paired by patient ID, and we used an FC > 1.5 and an FDR < 0.05 to define DEGs. When comparing negative visual responses to baseline, 11 genes were differentially expressed (10 up, 1 down) (Fig 2, *A*). For positive visual response versus baseline comparisons, 124 DEGs (76 up, 48 down) were identified. Of these 124 DEGs, 11 genes overlapped with the negative visual response versus baseline comparison (Fig 2, *B*). These changes may be the effect induced by the injection itself. Following removal of these 11 genes, 113 genes (66 up, 47 down) were found unique to a positive visual response to food injection (Fig 2, *B*; Table E2 in the Online Repository at www.jacionline.org). Upregulated genes were related to the cellular response to epidermal growth factor (*ERRFI1, SOX9, ID1, MYC, ZFP36L2*), ERK1 and ERK2 cascade (*ERRFI1, SOX9, IL1B, BMP2, MYC, DUSP6, CCN1, ZFP36L2, ATF3*), and cellular response to external stimulus (*PTGS2, HSPA8, SRF, CDKN1A, SOX9, IL1B, ATF3, NUAK2, CEBPB, FOSL1*) (Table E3 in the Online Repository at www.jacionline.org). There were no significantly enriched GO terms or KEGG pathways in the downregulated gene cluster. Interestingly, 1 of 113 genes was also differentially expressed when compared with a negative visual response (Fig 2, *A* and *B*).

Of the 113 genes that were unique to a positive visual response to food injection, 40 genes (34.5%) also had an FC of >1.5 in the positive versus negative visual response comparison but did not pass the FDR cutoff of 0.05 (Fig 3; Table E4 in the Online Repository at www.jacionline.org). Because of the exploratory nature of this study and because DEGs are sensitive to arbitrary cutoffs,[36] we continued further downstream analysis with this set of 40 genes. For ease, we refer to this set of 40 genes henceforth as early EoE genes.

TNFSF18 was the most robustly and significantly upregulated early EoE gene in both comparisons (12.35-fold for positive vs baseline and 18.27-fold positive vs negative) (Fig 3, *A*). Other upregulated early EoE genes were *CEBPB* (1.75-fold for positive vs baseline; 1.53-fold for positive vs negative), *IL1B* (3.26-fold for positive vs baseline; 2.83-fold for positive vs negative), *ENSG00000275216* (novel transcript affiliated with long noncoding RNA class) (6.36-fold for positive vs baseline; 2.5-fold for

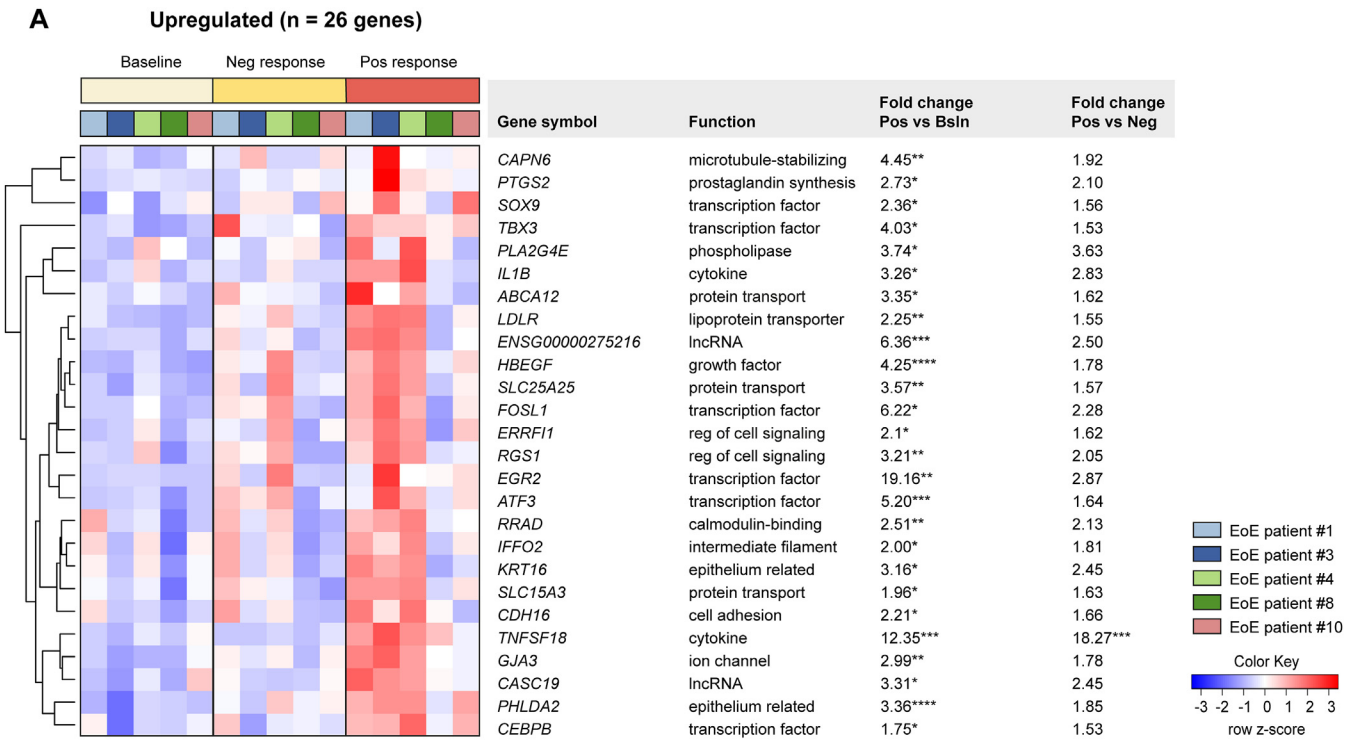

FIG 3. Early EoE genes. **(A** and **B)** Hierarchical clustering heatmap showing z scores for the 26 upregulated DEGs *(A)* and 14 downregulated DEGs *(B)* for each patient before injections (baseline), 20 minutes after a negative visual response to food injection (Neg response) and 20 minutes after a positive visual response to food injection (Pos response). Genes are shown on the right along with their function and FC for the indicated comparison. *Adjusted $P < .05$, **adjusted $P < .01$, ***adjusted $P < .001$, ****adjusted $P < .0001$. *lncRNA*, long noncoding RNA; *NA*, not available; *reg*, regulator.

positive vs negative), *PHLDA2* (3.36-fold for positive vs baseline; 1.85-fold for positive vs negative), and *SLC15A3* (1.96-fold for positive vs baseline; 1.63-fold for positive vs negative) (Fig 4, *A*). We confirmed increased expression of 3 of 6 genes (*CEBPB, IL1B,* and *TNFSF18*) by quantitative PCR (Fig 4, *B*). Plots for the remaining 20 upregulated early EoE genes and 14 downregulated early EoE genes are provided in Figs E1 and E2 (in the Online Repository at www.jacionline.org), respectively.

Early EoE genes are enriched in processes related to immune activation

To gain insight in the collective putative function of the genes that were activated in the early phase of the immune response to food allergens in EoE, we performed GO and KEGG enrichment analysis on the upregulated and downregulated early EoE genes separately. In the upregulated gene cluster (n = 26 genes) (Fig 3, *A*), GO analysis demonstrated gene expression related to immune activation, including the neuroinflammatory response

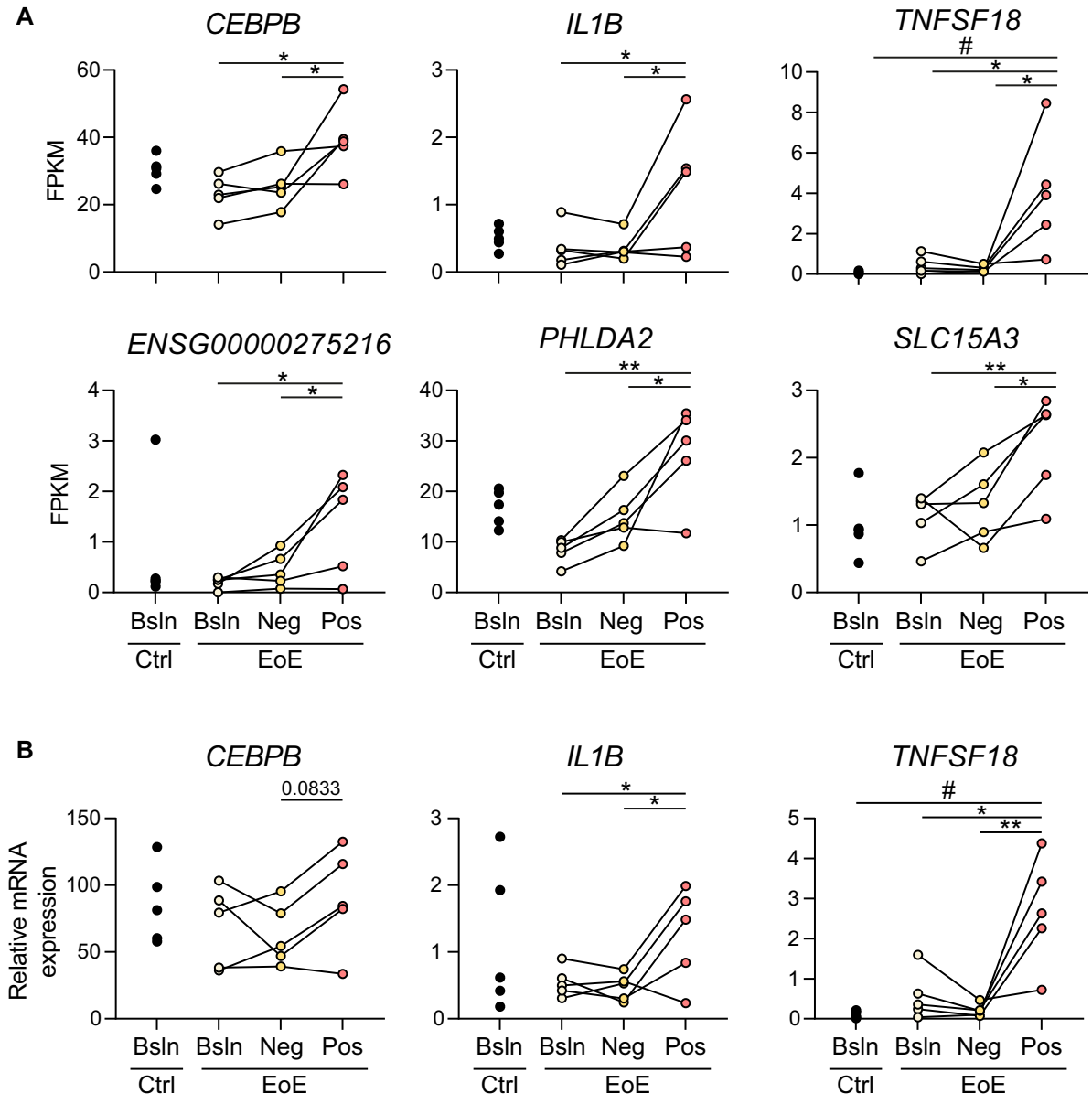

FIG 4. Gene expression pattern of 6 early EoE genes following a visual positive or a negative response to local challenge by food injection. **(A** and **B)** mRNA expression of *CEBPB, ENSG00000275216, IL1B, PHLDA2, SLC15A3,* and *TNFSF18* in esophageal biopsy specimens control subjects without EoE (Ctrl) at baseline (Bsln) and patients with EoE (EoE) at baseline and after a negative (Neg) or positive visual response (Pos) to food injection as measured by RNA sequencing *(A)* and RT-PCR *(B).* Data are presented as fragments per kilobase of gene model per million mapped reads (FPKM). **P* < .05, ***P* < .01, repeated measures one-way ANOVA and Bonferroni multiple comparisons test. #*P* < .05, Welch t test.

(*PTGS2, IL1B, LDLR*), the acute-phase response (*PTGS2, IL1B, CEBPB*), positive regulation of the inflammatory response (*PTGS2, TNFSF18, IL1B, LDLR, CEBPB*), cellular responses to external stimuli (*PTGS2, SOX9, IL1B, ATF3, CEBPB, FOSL1*), and cell population proliferation (*PTGS2, HBEGF, ERRFI1, TNFSF18, SOX9, IL1B, TBX3, ATF3, CEBPB, FOSL1, PHLDA2*). In addition, KEGG pathway analysis revealed enriched IL-17 signaling (*PTGS2, IL1B, CEBPB, FOSL1*), C-type lectin receptor signaling (*PTGS2, EGR2, IL1B*), and TNF signaling (*PTGS2, IL1B, CEBPB*). The GO terms and KEGG pathways associated with the early EoE genes, along with their fold enrichment and adjusted *P* values are shown in Table II. A complete list of enriched GO terms and KEGG pathways are provided in Table E5 (in the Online Repository at www.jacionline.org). In the downregulated gene cluster (n = 14 genes) (Fig 3, *B*), there were no enriched GO terms or KEGG pathways.

Esophageal epithelial cells are a potential source of the early EoE gene *TNFSF18*

TNFSF18, also known as *GITRL*, was the most prominently upregulated gene in the positive visual response versus baseline (12.35-fold) as well as the positive visual response versus negative visual response comparison (18.27-fold) in EoE patients

TABLE II. GO and KEGG profile of the upregulated early EoE genes (n = 26 genes)

Pathway database	GO term or KEGG pathway	Adjusted *P* value	Fold enrichment	Genes
GO BP	Neuroinflammatory response (GO: 0150076)	2.70×10^{-3}	66.9	*PTGS2*, ***IL1B***, *LDLR*
GO BP	Acute-phase response (GO: 0006953)	2.70×10^{-3}	64.3	*PTGS2*, ***IL1B***, ***CEBPB***
GO BP	Positive regulation of inflammatory response (GO: 0050729)	4.95×10^{-4}	30.6	*PTGS2*, ***TNFSF18***, ***IL1B***, *LDLR*, ***CEBPB***
GO BP	Cellular response to external stimulus (GO: 0071496)	2.70×10^{-3}	11.2	*PTGS2*, *SOX9*, ***IL1B***, *ATF3*, ***CEBPB***, *FOSL1*
GO BP	Regulation of cell population proliferation (GO: 0042127)	2.30×10^{-3}	4.9	*PTGS2*, *HBEGF*, *ERRFI1*, ***TNFSF18***, *SOX9*, ***IL1B***, *TBX3*, *ATF3*, ***CEBPB***, *FOSL1*, ***PHLDA2***
KEGG	IL-17 signaling pathway (hsa04913)	5.11×10^{-4}	33.8	*PTGS2*, ***IL1B***, ***CEBPB***, *FOSL1*
KEGG	C-type lectin receptor signaling pathway (hsa04625)	1.36×10^{-2}	20.2	*PTGS2*, *EGR2*, ***IL1B***
KEGG	TNF signaling pathway (hsa04668)	1.47×10^{-2}	17.08	*PTGS2*, ***IL1B***, ***CEBPB***

Fold enrichment is defined as the percentage of genes in the set of interest belonging to a term/pathway, divided by the corresponding percentage of genes in the background set that belong to the same term/pathway. Genes in bold are part of the 6 early EoE genes shown in Fig 4, *A*.
BP, Biological process.

who underwent intramucosal food injections (Fig 3, *A*, and Fig 4, *A*). To assess the potential cellular source of *TNFSF18*, we first explored a publicly available single-cell RNA-seq dataset of whole EoE esophageal biopsy specimens[37,38] and found that *TNFSF18* was enriched in differentiating epithelial cells compared with other esophageal cells in active EoE (Fig 5, *A*). To further investigate the epithelium as a potential source of *TNFSF18*, we stimulated EPC2-hTERT cells with the EoE-relevant cytokines IL-13[39] and TNF-α (Fig 5, *B*).[9,40,41] IL-13 and TNF-α costimulation induced a robust increase in *TNFSF18* mRNA expression by EPC2-hTERT cells in a time-dependent manner compared with IL-13 and TNF-α alone (Fig 5, *C*). TNF-α, but not IL-13, stimulation alone also significantly induced *IL1B* and *SLC15A3* mRNA expression after 4 hours (Fig E4 in the Online Repository at www.jacionline.org). Furthermore, some of the morphological changes in the esophageal mucosa following food injections might be driven by mast cell activation.[24,37,42] We therefore also tested supernatant from activated primary human mast cells (1.5×10^6 cells/mL)[43] on *TNFSF18, IL1B, SLC15A3, CEBPB,* and *PHLDA2* mRNA expression in EPC2-hTERT cells, but no effect was observed (data not shown).

DISCUSSION

In this study, we analyzed transcriptomic profiles of esophageal biopsy specimens from adult patients with EoE who underwent esophageal challenge by intramucosal food injections to characterize gene expression signatures and functional processes associated with the acute esophageal response to a specific food. We identified 113 genes that were unique to a positive visual response to intramucosal food injections, of which 40 genes were dysregulated by more than 1.5-fold compared with a negative visual response to intramucosal food injections. These early EoE genes were enriched in proinflammatory processes, such as the acute-phase response and cellular response to external stimuli. Of the early EoE genes, *TNFSF18* (also called *GITRL*), a member of the TNF superfamily best studied for costimulatory effect on T cells, was most highly induced following a positive visual response to food injection compared with a negative visual response (18-fold) and baseline (12-fold). Interestingly, *TNFSF18* appears to play a role in other atopic conditions, such as asthma[44] and atopic dermatitis.[45,46] Finally, we show that esophageal epithelial cells may be an early source of *TNFSF18*. The data presented herein for the first time provide insight into the early transcriptomic changes that are associated with an acute mucosal response to food allergens in EoE.

The increasing knowledge of pathogenic pathways and cytokines in EoE derives mostly from bulk or single-cell RNA-seq studies of esophageal biopsy specimens collected during active and inactive disease.[13,17,19,37,38] However, to date, there have been no studies reported that performed RNA-seq on esophageal tissue collected just after exposure to food. A major strength of this study is that we could characterize changes in gene expression that occurred during an acute response to food by profiling the transcriptomes of patients with EoE before and shortly after esophageal challenge by intramucosal food injections. Rather than characterizing the active EoE transcriptome, our study design allowed us to leverage data collected at different time points (baseline vs after injection) and between esophageal responses (negative vs positive) within the same patient, increasing power by reducing bias due to interindividual variability. It should be noted that EoE is patchy, resulting in differences in cellular composition of the biopsy specimens from the same patient. By comparing a positive visual response biopsy specimen with both baseline and negative visual response biopsy specimens, we aimed to reduce the effect of patchiness on the precision of the analysis.

The current transcriptome study provides a comprehensive molecular map of immune alterations that occurred in the esophagus of adult patients with EoE following esophageal challenge by intramucosal food injections. We identified early EoE genes that showed significant changes in expression in the early phase of the immune response to food allergens in EoE. The observed expression signatures were involved in proinflammatory processes, such as the acute-phase response, cellular response to external stimuli, and regulation of cell population proliferation. These functional categories are similar to those identified in a dynamic transcriptome study that characterized changes in peripheral blood samples during an allergic response to peanut.[47]

Six early EoE genes (*ENSG00000275216, PHLDA2, SLC15A3, IL1B, CEBPB,* and *TNFSF18*) demonstrated increased

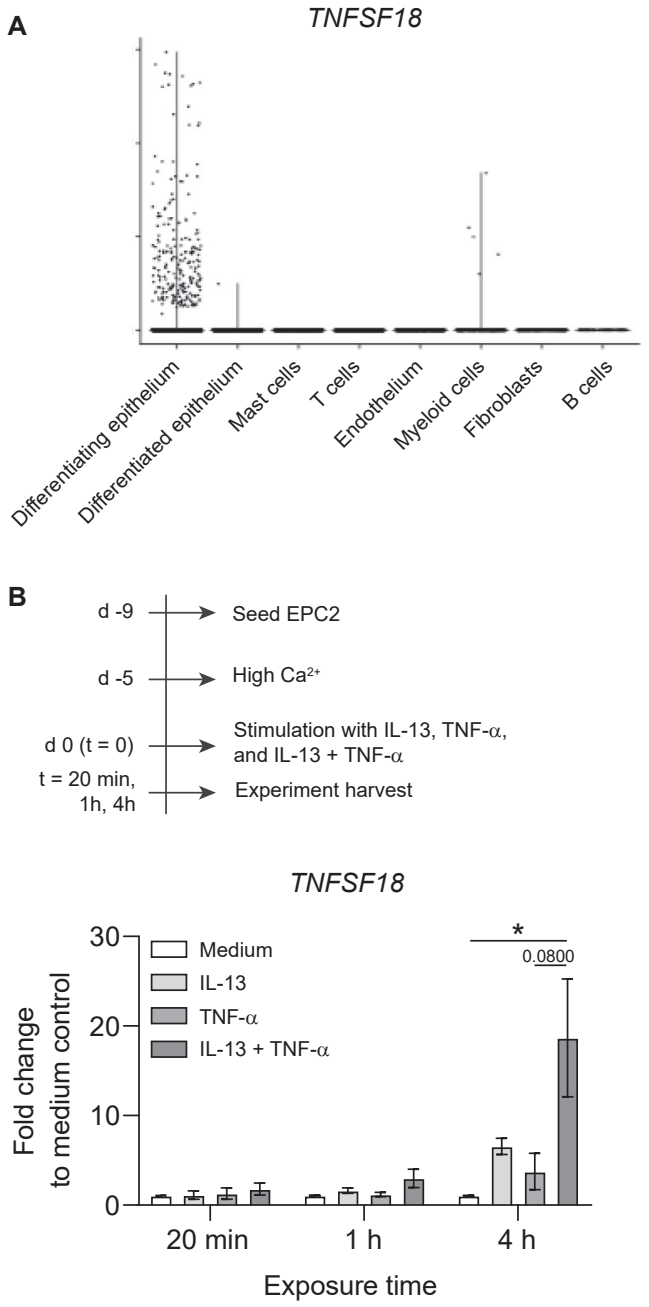

FIG 5. *TNFSF18* expression in esophageal epithelial cells. **(A)** *TNFSF18* mRNA expression in different esophageal cell populations from patients with active EoE (n = 5), as identified by single-cell RNA-seq of whole esophageal biopsy specimens.[37,38] **(B)** Schematic overview of the *in vitro* EPC2-hTERT (EPC2) stimulation experiments *(top)*, and *TNFSF18* mRNA expression in EPC2 cultures after 20 minutes, 1 hour, and 4 hours of exposure to the indicated cytokines *(bottom)*. Data are presented as mean ± SEM from n = 3 independent experiments performed with 2 technical replicates per condition. **P* < .05, by one-way ANOVA and Bonferroni multiple comparisons test.

expression triggered by local food allergen exposure. The role of *ENSG00000275216* (novel transcript affiliated with long noncoding RNA class) in immune responses is not established. Though IgE cross-linking on human mast cells is accompanied with increased expression of *PHLDA2*,[48] its functional role in allergic inflammation is not clear. Expression of *SLC15A3* has been shown in monocytes, where it has a role in driving virus-induced production of type I and III interferons.[49] *IL1B*, *CEBPB*, and *TNFSF18* have established and validated roles in allergic inflammation. *IL1B* encodes the proinflammatory cytokine IL-1β that is produced by a variety of immune cells, including dendritic cells, macrophages, and B cells as well as nonimmune cells such as keratinocytes.[50] In addition, IL-1β has been implicated in the pathogenesis of atopic dermatitis and asthma[51-53] and drives mast cell hyperactivation in atopic dermatitis–like inflammation in mice.[54] *CEBPB* encodes a transcription factor that regulates genes involved in proinflammatory responses[55] and was found to be upregulated in esophageal eosinophils in IL-13–induced experimental EoE.[56] Interestingly, both *IL1B* and *CEBPB* were increased in peripheral blood leukocytes from subjects admitted to the emergency department with anaphylaxis.[57] *TNFSF18*, which was most prominently upregulated during an acute response to food injection, encodes the TNFSF18/GITRL protein that occurs in transmembrane and soluble forms.[58] While TNFSF18 is expressed on professional antigen-presenting cells, including dendritic cells, macrophages, and B cells, as well as nonprofessional antigen-presenting cells such as endothelial and epithelial cells, its receptor (TNFRSF18/GITR) is mainly expressed on effector and regulatory T cells, but also ILC2s.[58-60] Ligation of TNFRSF18 by either anti-TNFRSF18 antibodies or its natural ligand TNFSF18 typically results in the activation or enhancement of the immune response[61] and has been shown to stimulate effector T cells and attenuate regulatory T cell–mediated suppression.[62-66] Cosignaling between TNFRSF18 and IL-33 receptor promotes human ILC2 expansion and expression of type 2 cytokines IL-9, IL-5, and IL-13.[60] Upon transmembrane TNFSF18-TNFRSF18 interaction, TNFSF18 can transduce bidirectional signals, of which most have a proinflammatory function.[59,67] So, TNFSF18 does not merely function as a trigger protein for TNFRSF18, but also modulates activity of the cells that express TNFSF18 itself.[68]

TNFSF18-TNFRSF18 interactions have a critical role in allergic inflammation. Several *in vivo* studies using murine models of allergic asthma have demonstrated a role for TNFSF18 in promoting T_H2 cell differentiation and effector functions and in enhancing lung allergic responses by inducing T_H2 cell and ILC2 activity.[44,60,66,69,70] In EoE, *TNFSF18* expression is increased in esophageal biopsy specimens and fibroblasts.[71] A role in EoE pathogenesis was recently attributed to another TNF superfamily member, TNFSF14/LIGHT,[71-73] as its overexpression induced a proinflammatory phenotype in fibroblasts in EoE,[72] while its deficiency protected mice from developing EoE-like inflammation.[73]

Here, we demonstrated that *TNFSF18* mRNA expression can be induced in esophageal epithelial cells by IL-13 and TNF-α costimulation. This is in line with a previous report on human epidermal keratinocytes,[45] which can form stratified squamous epithelia similar to esophageal epithelial cells. Interestingly, TNFSF18 expression is increased in keratinocytes of acute skin lesions of patients with atopic dermatitis.[45] Ligation of TNFSF18 expressed on human keratinocytes induced an increase in expression of the proinflammatory cytokine IL-8 and T-cell chemokine CCL27.[74] It is interesting to speculate that the food-induced increase in *TNFSF18* expression in the esophageal epithelium may promote the signaling potential between the epithelium and TNFRSF18-expressing T cells and ILC2s, resulting in the production of a plethora of proinflammatory mediators from

epithelial cells, proliferation of T cells, and activation of ILC2s. The source of *TNFSF18* in EoE still requires further study. While the esophageal epithelium is indeed one of the primary drivers of EoE pathogenesis,[75] and we identified *TNFSF18*-expressing epithelial cells, we cannot rule out that the increase in *TNFSF18* mRNA levels on food challenge comes from another cellular source, which would require single-cell RNA-seq. If *TNFSF18* indeed plays an initial and essential role in the early phase of the food-induced immune response in EoE, blockade of the TNFSF18-TNFRSF18 pathway may provide a new therapeutic target for EoE as it may become in asthma.[44] Future mechanistic studies should therefore aim at establishing the function of the TNFSF18-TNFRSF18 pathway in EoE.

This study has limitations. First, this exploratory study was conducted in a small cohort, and future studies in larger cohorts should be performed to confirm our findings. Second, esophageal biopsy specimens were collected from the injection sites 20 minutes after intramucosal food injections. While changes in gene expression can be measured within 2 minutes after stimulation,[76] biopsy specimens taken at a later time point may have provided a broader insight into the immune mechanisms underlying a mucosal response to food. However, due to the invasiveness of the procedure and the discomfort that several patients experienced, it was ethically not possible to prolong the endoscopy to collect biopsy specimens at a later time point. It would be interesting for future studies to profile the dynamic transcriptome using biopsy specimens collected at multiple later time points following exposure to food. Third, the way the esophageal mucosa is exposed to food allergens in this study is different from natural exposure. Via intramucosal injections, we may have bypassed an initial response to the allergen by the epithelium, an active participant in the immune system.

In conclusion, we show that esophageal challenge by intramucosal food injections in adult patients with EoE triggers the expression of genes that are associated with processes related to immune activation. Our study identifies *TNFSF18/GITRL* as the most upregulated gene during an acute response to food injections. As such, TNFSF18 may mediate interactions between TNFSF18-expressing cells including esophageal epithelial cells and TNFRSF18/GITR-expressing cells including T cells and ILC2s during an acute mucosal response to food in patients with EoE to promote inflammation. Further studies on a possible role of *TNFSF18* in acute mucosal responses to food and EoE pathogenesis are needed to determine if the TNFSF18-TNFRSF18 pathway may be a new therapeutic target for EoE.

DISCLOSURE STATEMENT

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Key messages

- **Intramucosal food injections in the esophagus induced an acute transcriptome profile enriched in genes functionally involved in immune activation.**
- ***TNFSF18/GITRL* was the most prominently upregulated gene 20 minutes after food exposure by intramucosal injection and can be induced in esophageal epithelial cells by inflammatory triggers.**

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