Published in final edited form as:

J Allergy Clin Immunol. 2017 September ; 140(3): 738–749.e3. doi:10.1016/j.jaci.2016.11.042.

Profound Loss of Esophageal Tissue Differentiation in Eosinophilic Esophagitis

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Abstract

Background—A key question in the allergy field is to understand how tissue specific disease is manifested. Eosinophilic esophagitis (EoE) is an emerging tissue specific allergic disease whose pathogenesis remains unclear.

Objective—Herein, we tested the hypothesis that a defect in tissue specific esophageal genes is an integral part of EoE pathogenesis.

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Conflict of interest: T. Wen's institution has received an Aimmune research grant for other works, and they have an EDP patent owned by Cincinnati Children's Hospital Medical Center (CCHMC). J.M. Caldwell, M. Rochman, and M.E. Rothenberg are coinventors of patents owned by CCHMC. M. E. Rothenberg's institution has received a grant from the NIH for this work; they have grants for other works from a US-Israel Binational Grant and PCORI; they have patent, inventorship, and ownership by CCHMC; he has received consultancy fees from Celgene, Gennetech, Immune Pharmaceuticals, NKT Therapeutics, and PulmOneTherapeutics; he has received payment for lectures from Merck; and he has stock options with PulmOne, NKT Therapeutics, and Immune Pharmaceuticals. The rest of the authors declare that they have no relevant conflicts of interest.

Author contributions

M.R. and J.T. performed experiments, data analysis and wrote the paper; N.P.A., J.M.C., B.P.D., J.K.R, K.K, T.W, M.C.B, C.E.M and J.D.S. performed experiments and data analysis; M.E.R. supervised the study.

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Methods—We interrogated the pattern of expression of esophagus-specific signature genes derived from the Human Protein Atlas in the EoE transcriptome and in EPC2 esophageal epithelial cells. Western blot and immunofluorescence were used for evaluating expression of esophageal proteins in control and active EoE biopsies. Whole-exome sequencing (WES) was performed to identify mutations in esophagus-specific genes.

Results—We found that ~39% of the esophagus-specific transcripts were altered in EoE, with ~90% being downregulated. The majority of transcriptional changes observed in esophagus-specific genes were reproduced *in vitro* in esophageal epithelial cells differentiated in the presence of IL-13. Functional enrichment analysis revealed keratinization and differentiation as the most affected biological processes, and identified IL-1 cytokines and serine peptidase inhibitors (SERPINs) as the most dysregulated esophagus-specific protein families in EoE. Accordingly, EoE biopsies evidenced a profound loss of tissue differentiation, decreased expression of keratin 4 and cornulin and elevated expression of keratin 5 and 14. Whole-exome sequencing of 33 unrelated EoE cases revealed 39 rare mutations in 18 esophagus-specific differentially expressed genes.

Conclusions—A tissue-centered analysis has revealed a profound loss of esophageal tissue differentiation (identity) as an integral and specific part of the pathophysiology of EoE, and implicated protease- and IL-1–related activities as putative central pathways in disease pathogenesis.

Keywords

eosinophilic esophagitis; IL-1 cytokines; protease activity; IL-13; differentiation; whole-exome sequencing; mutations

Introduction

Recent advances in human proteomics and transcriptomics have led to identification of tissue-specific genes that confer molecular identity to individual tissues, as highlighted by the Human Protein Atlas project that combines tissue-specific proteome data with global gene expression analysis for over 40 human tissues.¹ These data sets allow examination of the contribution of the tissue-specific genes to disease pathogenesis, constituting a novel, tissue-centered approach for diagnostics and potentially for predictive purposes.

Eosinophilic esophagitis (EoE) is a chronic allergic inflammatory disease characterized by eosinophil-predominant, tissue-specific, mucosal inflammation. The disease is mediated in large part by an immune response to food allergens. This immune response involves the interaction of the innate immunity (epithelium and eosinophils) with a strong T helper type 2 (Th-2) adaptive immune response characterized by IL-13 overproduction, resulting in a marked alteration in esophageal gene expression. Notably, IL-13 directly stimulates the epithelium to produce a large subset of the EoE-associated transcriptome, including the eosinophil chemoattractant and activating factor CCL26 (eotaxin-3), thus propagating the inflammatory loop.^{2, 3}

Esophageal tissue integrity has a critical role in disease pathogenesis,^{4–10} as illustrated by profound dysregulation of the epidermal differentiation complex (EDC) genes¹¹ and

decreased expression of desmoglein 1 (*DSGI*) in active EoE and in IL-13–stimulated esophageal epithelial cells.⁴ In line with these findings, genes for structural proteins including keratin 6C, keratin 32 and the EDC gene cornulin (CRNN), are among the most highly expressed genes in the homeostatic esophagus on the basis of the Human Protein Atlas. Recent genome-wide association study (GWAS) analyses identified the cysteine protease calpain 14 (*CAPN14*), another highly expressed gene in the homeostatic esophagus, as one of the strongest genetic risk factors associated with disease susceptibility,^{12, 13} suggesting that protease activity is critical for disease pathophysiology. Indeed, CAPN14, as well as serine peptidase inhibitor SERPINB13, were upregulated in EoE biopsies, and dysregulation of CAPN14 in esophageal epithelial cells resulted in impaired barrier function of differentiated epithelium.¹⁴

Herein, we interrogated changes in the esophagus-specific transcriptome defined by the Human Protein Atlas (297 genes expressed > 5 fold higher in esophagus vs. any other human tissue¹) in EoE. We identified 117 esophagus-specific genes that were significantly dysregulated in EoE. Remarkably, ~90% of these genes were downregulated, demonstrating a profound loss of esophageal tissue gene signature in the allergic inflamed esophagus. Functional analysis of the esophagus-specific genes altered in EoE revealed loss of esophageal differentiation, identified proteolytic activity as a central pathway impaired in the diseased esophagus and implicated genes from the SERPIN and IL-1 families as those most significantly affected of the tissue-specific genes in EoE. Quantification of IL-36α, SERPINs B13 and B4 and CRNN proteins in control and inflamed esophageal biopsies corroborated transcriptional data. Transcriptional changes observed in the inflamed esophagus were largely reproduced in esophageal cells treated with IL-13 *in vitro*, which showed an impaired epithelial cell differentiation program. Whole-exome sequencing (WES) analysis of 33 unrelated EoE cases identified 39 rare mutations in 18 esophagus-specific genes that were transcriptionally altered in the disease. Functional enrichment analysis identified proteolytic activity and tissue differentiation as the most significant pathways associated with these genes. These data substantiate a potential functional link between the esophageal transcriptome and EoE pathogenesis. Collectively, our study reveals profound loss of esophageal epithelial differentiation as a hallmark of EoE and implicates protease- and IL-1–related activities as putative, key, tissue-specific pathways involved in the disease. We propose that the loss of the esophagus-specific molecular signature in EoE is a disease-specific process that has potential value for predictive medicine, diagnostics and treatment of this disease.

Materials and Methods

Gene definitions

Definitions related to specificity of expression in the esophagus were from the Human Protein Atlas (<http://www.proteinatlas.org/humanproteome/esophagus>; Accessed May 2016.). Tissue-enriched genes are expressed in the esophagus at least 5-fold higher than in any other tissue transcriptionally profiled by the Human Protein Atlas, group-enriched genes are expressed at least 5-fold higher in the group of 2–7 tissues, and tissue-enhanced genes are expressed at least 5-fold higher in the esophagus compared to the average of expression

in all tissues. Post acceptance updates to the Human Protein Atlas may alter the number of tissue-specific (and esophagus-specific) genes from values presented herein.

Expression data and whole-exome sequencing

Publically available expression data were used for esophageal biopsies of patients with EoE or controls³ (GSE58640), antrum biopsies of patients with EG or controls¹⁵ (GSE54043), TE-7 cells⁴² (GSE57637) and EPC2 ALI cultures²¹ (GSE58640, GSE65335). There was no statistical difference (Mann-Whitney U-test $p = 0.26$) in ages between patients in EoE and control groups used for RNA sequencing analysis.³ Lists of esophagus- and stomach-specific genes were obtained from the Human Protein Atlas (<http://www.proteinatlas.org>). Gene ontology (GO) enrichment analysis, which uses statistical methods to determine functional pathways and cellular processes associated with a given set of genes, was performed using ToppGene suite (<https://toppgene.cchmc.org/>¹⁶); Venny (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>) was used to intersect gene lists; Cluster 3.0 was applied for clustering genes using Euclidean distance and average linking parameters, and Java TreeView was used for visualization of heat maps (<http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm>). WES analysis was performed and analyzed as in Patel ZH *et al.*²⁴ Briefly, only variant calls that had a read depth greater than 15 and a genotype quality score greater than 20 were included. In addition, variants were removed that had a minor allele frequency greater than 1% in the general population (based on 1000 genomes project (<http://www.1000genomes.org/>) or the NHLBI Exome Sequencing Project (<https://esp.gs.washington.edu>) and were required to alter the amino acid sequence of protein.

Western blot analysis

Proteins from control and EoE biopsies were isolated by Trizol, subjected to electrophoresis on a 4–12% protein gel and probed with anti-mouse HSP90 (BD Bioscience), goat anti-IL-36 α (R&D), rabbit anti-SERPINB13 (Sigma Aldrich), goat anti-cornulin (AF3607, R&D) and mouse anti-SERPINB4 (Santa Cruz) antibodies. Secondary IRDye-conjugated antibodies were from Licor. Quantification of signal was performed using Image Studio Lite software (http://www.licor.com/bio/products/software/image_studio_lite/). Signal from the tested proteins was normalized to the signal from the loading control (HSP90) following normalization to the level of the protein in one of the control samples. Statistical analysis was performed in GraphPad software.

Air-liquid interface (ALI) culture system

The ALI differentiation protocol was performed essentially as described^{4, 21}. Briefly, EPC2 immortalized esophageal epithelial cells were seeded onto semi-permeable membranes (0.4 μ m) and grown to confluence in standard low-calcium medium (KSFM with pituitary extract and EGF at 1 ng/ml, 0.09 mM calcium concentration). Epithelial differentiation was induced by the addition of KSFM with high calcium (1.8mM final concentration) over the course of five days (initial differentiation). Medium was replaced in both chambers every other day. Stratification was induced by removing the media from the upper chamber and exposing the cells to the air interface for a period of 5–6 days (terminal differentiation). Cells remained exposed to high calcium medium in the bottom chamber throughout terminal differentiation. Treatment with IL-13 (100 ng/mL) in the lower chamber started at the first day of exposure

to the air interface (day 8), with fresh media with IL-13 added every other day. In some experiments, cultures were treated with IL-1 α (10 ng/ml) for the last 2 days of ALI culture. At the last day of ALI culture, cells were collected for analysis.

Immunofluorescence analysis

Immunofluorescence staining was performed as previously described⁴ on at least four distal esophageal biopsies from control individuals, distal esophageal biopsies with active inflammation from patients with active EoE, or proximal esophageal biopsies without active inflammation from the same patients with active EoE. The average ages of EoE patients and control individuals were 13 and 11 years, respectively. Patient groups were also controlled for race and sex. The following primary antibodies were used at a final concentration of 1 μ g/mL: anti-keratin 14 (PRB-155P, Covance), anti-keratin 5 (ab24647, Abcam), anti-keratin 4 (HPA034881, Sigma), or anti-cornulin (AF3607, R&D). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Slides were blocked with phosphate-buffered saline with 10% goat or donkey serum. Secondary antibodies (1:500 dilution) used were donkey anti-rabbit Alexa Fluor 647 (A31573, Life Technologies), goat anti-rabbit Alexa Fluor 647 (A21244, Life Technologies), or donkey anti-goat Alexa Fluor 488 (A11055, Life Technologies). Imaging was performed with a Nikon A1 inverted confocal microscope.

Results

Specific loss of esophageal signature genes in EoE

To test the hypothesis that esophagus-specific genes contribute to EoE pathogenesis, we intersected genes transcriptionally altered in EoE³ with various organ-specific transcriptomes obtained from the Human Protein Atlas, including esophagus, salivary gland, gastrointestinal tract organs, kidney, lung, skin and testis. Genes in the transcriptomes are classified as tissue-enriched (expressed at least 5 fold higher in the interrogated tissue than in any other tissue), group-enriched (at least 5 fold higher expression in the group of tissues compared to other tissues) and tissue-enhanced (at least 5 fold higher expression in the interrogated tissue compared to average level in all tissues). We found that the percentage of esophagus-specific genes altered in EoE was at least two fold higher than that of genes from other organ-specific transcriptomes (Figure 1A). From 297 genes whose expression is specifically enriched in the esophagus relative to other tissues, we found 117 genes overlapping with the EoE transcriptome (designated as Eso-EoE genes), which represent 39% of esophageal signature genes and 7% of genes altered in EoE (Figure 1B, Suppl. Table 1). Notably, 89% of Eso-EoE genes (104 of 117 genes) were significantly downregulated in the esophageal tissue of patients with EoE (Figure 1B). Of the genes most highly expressed at the mRNA level in the homeostatic esophagus, 12 genes were expressed ~10–94 fold higher in the esophagus compared to other interrogated tissues. Of these, only *CAPN14* and *SERPINB13* were significantly upregulated in EoE (Figure 1C). Overall, for the 117 tissue-enriched, tissue-enhanced and group-enriched genes, only 13 genes were significantly upregulated in EoE (Figure 1B). A similar comparison between stomach-specific signature genes and the eosinophilic gastritis (EG) transcriptome¹⁵ identified only 16 overlapping genes, representing 8% of the stomach transcriptome and approximately 1.5% of the transcripts dysregulated in EG (Figure 1D). These data suggest that loss of the tissue-

specific molecular signature of the esophagus is an integral and specific part of the pathophysiology of EoE.

Functional enrichment analysis of Eso-EoE genes

By performing functional enrichment gene ontology (GO) analysis¹⁶ of Eso-EoE genes, we identified endopeptidase inhibitor activity and keratinization as the most profoundly impaired molecular functions and biological processes ($p < 10^{-9}$ and $p < 10^{-14}$, respectively; Figure 2A, B). Accordingly, a number of SERPIN-related genes were upregulated (*SERPINB2*, *B3*, *B4*, *B13*), whereas genes from the serine protease inhibitor Kazal-type (SPINK) family were downregulated (*SPINK5*, 7, 8). Notably, increased expression of SERPINS, specifically SERPINE1 (PAI-1), has been previously reported in EoE and asthma and correlated with disease severity.^{17, 18} The downregulated genes also included the differentiation markers keratin (*KRT6B*) and involucrin (*IVL*) and the small proline-rich proteins (SPRRs) that comprise members of the EDC previously implicated in EoE pathogenesis.¹¹ Transglutaminases 1 and 3 (*TGM1*, *TGM3*), associated with pathogenesis of the rare cornification disease autosomal recessive congenital ichthyosis¹⁹, were also downregulated in EoE. In line with dysregulation of structural epithelial genes, the most significantly affected human phenotypes were abnormality of keratinization, epidermal thickening, hyperkeratosis and skin inflammation (Figure 2C). These findings revealed a profound loss of esophageal tissue differentiation in EoE and a potentially critical role of esophagus-specific proteases in supporting the functional integrity of the epithelial barrier of the esophagus.

We next analyzed the properties of Eso-EoE genes on the basis of the common protein domains. The two top groups of genes most highly enriched by this analysis were serine protease inhibitors (SERPINS; *SERPINB2*, *B3*, *B4*, *B13*) and IL-1 family members (*IL36A*, *IL36RN*, *IL1A*, *IL1RN*). SERPINS were upregulated ($p < 10^{-7}$), whereas IL-1-related genes were significantly downregulated in EoE ($p < 10^{-5}$; Figure 2D). Other protein domains enriched in Eso-EoE genes were SPINKs, keratins, members of the arginine deiminase family (*PADI1*, 3) and transglutaminases (*TGM1*, 3) (data not shown).

Eso-EoE genes are regulated during esophageal epithelial differentiation

We aimed to recapitulate the inflammatory process in a cell system *in vitro* to test whether esophagus-specific changes in transcription were driven by epithelial rather than a tissue-based, mixed cellular response. For this purpose, we utilized air-liquid interphase (ALI) culture of an esophageal epithelial cell line, EPC2 cells that undergoes squamous cell differentiation.²⁰ Submerged EPC2 cells were grown in low calcium (0.09 mM) and subjected to initial differentiation by exposure to a high concentration of calcium (1.8 mM) followed by terminal differentiation at the ALI with and without IL-13 stimulation²¹ (Figure 3A). Initial differentiation with high calcium triggered dramatic transcriptional changes in submerged culture with 3146 genes changing expression. Overlapping these genes with Eso-EoE genes revealed that 90 Eso-EoE genes (77%) were transcriptionally altered during initial differentiation (Figure 3B), with the vast majority being upregulated in the initial differentiation in submerged culture but downregulated in the biopsies of patients

with EoE (Figure 3B, heat map). These results demonstrate the biological relevance of ALI EPC2 culture for studying esophageal epithelial differentiation.

IL-13 impacts the expression of Eso-EoE genes

We assessed the effect of IL-13 under conditions of terminal differentiation of epithelial cells at ALI culture (Figure 4A). We have previously shown that terminal differentiation of EPC2 cells in the presence of IL-13 leads to altered expression of epithelial differentiation genes.²¹ We identified 68 Eso-EoE genes (61% of Eso-EoE genes) affected in ALI culture: expression of 32 genes was altered under all conditions (Figure 4A, Group 1), 8 genes were altered in response to IL-13 stimulation but not by differentiation (Figure 4A, Group 2), and 28 genes were affected by the differentiation process but not by IL-13 (Figure 4A, Group 3). The gene expression pattern of Eso-EoE genes in ALI cultures differentiated in the presence of IL-13 became strikingly similar to that of the biopsies of patients with EoE (32 genes, Group 1). For example, *CAPN14*, *SERPINB3* and *SERPIN4* were upregulated in both IL-13—treated ALI cultures and biopsies, whereas *SPINK5* and genes from IL-1 and small proline-rich protein (SPRR) families were downregulated in both. We observed similar patterns of gene expression in biopsy and in ALI cultures differentiated in the presence of IL-13 independently of whether the genes were significantly affected by the differentiation process (8 genes, Group 2). The only exception was *SERPINB2*, which was downregulated in ALI culture. Epithelial differentiation led to changes of 28 genes that were refractory to IL-13 treatment (Group 3). Notably, expression of 16 genes from this group inversely correlated with expression in biopsies: the genes were upregulated in differentiated ALI culture but downregulated in the disease. We further delineated expression of IL-1 and *SERPINB* gene families in ALI system compared to active EoE biopsies. Consistent with other Eso-EoE genes, the expression pattern for these genes was reproduced in ALI culture for 5 out of 8 genes with IL-1 family members downregulated and *SERPINs* upregulated in IL-13—treated cells (Figure 4B).

In light of downregulated expression of *IL1A* in EoE biopsies and ALI cultures, we tested whether replenishment of IL-1 α would reverse gene expression in IL-13—treated cells subjected to ALI culture. Addition of IL-1 α to the cells subjected to ALI either alone or in the presence of IL-13 had no effect on the integrity of the culture, as measured by TEER, and did not reverse expression of the prototypic IL-13 target genes *CCL26* and *SERPINB4* (Supplemental Figure 1).

Additionally, in order to rule whether that this dysregulation of Eso-EoE genes is a common feature of esophageal disease in general, we assessed the changes in expression of Eso-EoE genes in gastroesophageal reflux disease (GERD), which also has epithelial damage and basal zone hyperplasia. None of the 9 Eso-EoE genes tested had dysregulated expression in GERD (Figure 4C). Collectively, these results indicate that the downregulation of esophageal signature genes in EoE is likely reflective of an intrinsic defect in esophageal epithelial cell differentiation in response to IL-13.

Quantifying Eso-EoE proteins in esophageal biopsies

We sought to assess levels of expression of Eso-EoE proteins including structural proteins, proteins related to proteolytic activity and IL-1 cytokines in the lysates of esophageal biopsies isolated from unaffected controls and patients with active EoE (minimum esophageal eosinophil count of at least 15 eosinophils/high-power microscopic field (hpf)). Following functional enrichment analysis, we focused on serine protease inhibitors SERPINB4 and B13, structural protein CRNN, and IL-1 cytokine family member IL-36α. Western blot analysis revealed significant changes in the levels of all tested proteins, which paralleled gene expression patterns (Figure 5A, B), supporting changes in the differentiation, protease activity and IL-1 cytokines in the esophageal tissue in EoE.

Expression of epithelial differentiation markers in the biopsies of patients with active EoE

Expression of genes related to keratinocyte differentiation was most significantly enriched in the Eso-EoE gene set; all of these genes were downregulated in EoE. We therefore assessed the differentiation status of epithelial tissue in the biopsies of patients with active EoE. We applied immunofluorescence to test expression of keratins as common markers of differentiation, as well as CRNN, a member of the EDC specifically expressed in esophagus (Figure 1B). In the biopsies of patients with active EoE, we observed increased expression of keratin 5 and 14 linked to undifferentiated epithelium and a dramatic loss of keratin 4 associated with terminally differentiated tissue²³ (Figure 5C–E). Esophagus-specific structural protein CRNN was highly expressed in the suprabasal layers of normal esophageal tissue and was downregulated in EoE following expansion of the basal zone of the epithelium (Figure 5F). Furthermore, the increased expression of basal keratins and decreased expression of differentiated keratins does not occur throughout the entire length of the esophagus, but rather in areas of active inflammation (compare Fig 5, C–F middle and bottom rows). Collectively, these data are consistent with marked loss of differentiation of the esophageal tissue in EoE.

Whole-Exome Sequencing (WES) analysis of Eso-EoE genes

Since loss of esophagus-specific genes appeared to be a hallmark of EoE, we reasoned that the disease might be associated with an increased burden of rare, damaging mutations in Eso-EoE genes. To test this hypothesis, we performed WES of 33 unrelated EoE cases to identify variants with the potential to alter the biological function of any of the proteins in the list of the 117 Eso-EoE genes. We only considered rare variants (less than 1% minor allele frequency based on public databases) that passed a number of quality control measurements²⁴ and altered the amino acid sequence (non-synonymous, gaining or losing a stop codon, altering the open reading frame, disrupting the initiation methionine codon, or altering splicing). We identified 39 variants that were located in 18 genes that met these criteria (Table 1). None of the patients had more than one rare variant in any particular gene. In addition, we assessed the number of genes with rare variants present in individual patients with EoE. Using the rare variants in Table 1, we identified 12 cases carrying only 1 gene, 7 cases carrying 2 genes, and 6 cases carrying 3 genes (data not shown). Among the genes identified as mutated in patients with EoE were those of the protease inhibitors SERPINB3 and SPINK5, CAPN14 and structural protein KRT6B. Interestingly, mutations in a multi-

subunit chloride channel gamma-aminobutyric acid (GABA) A receptor, π (GABRP), which was primarily studied in central nervous system, were identified in 4 out of 33 patients with EoE. Notably, functional enrichment analysis of these genes¹⁶ identified the most significant molecular function as serine-type endopeptidase inhibitor activity ($p=8.6\times 10^{-5}$) and biological processes involved in epidermal cell differentiation ($p=1.2\times 10^{-5}$). In order to test whether the number of mutations correlated with disease severity, we analyzed the number of eosinophils per hpf in the biopsies of the patients with different numbers of variants. We were unable to stratify patients with EoE based on the number of variants, probably due to the small number of patients in the groups (data not shown). Collectively, the WES data supported the view that there is a key role for esophagus-specific genes in esophageal differentiation and EoE pathogenesis.

Discussion

Herein we tested the hypothesis that dysregulation of esophagus-specific genes is an integral part of EoE pathogenesis. By comparing the esophageal gene signature with the transcriptome of the esophagus of EoE patients,³ we identified that ~39% of esophageal genes were altered in EoE (designated Eso-EoE genes); the vast majority of these genes were downregulated in the disease. Functional enrichment analysis of Eso-EoE genes revealed major alterations in keratinization and differentiation, protease-related activities and IL-1–related genes. We observed that the expression pattern of Eso-EoE genes from the biopsies of active EoE patients and in ALI cultures differentiated in the presence of IL-13 were remarkably similar, providing evidence that loss of esophageal differentiation does not merely reflect a change in the cellular constituents or expansion of the basal epithelial zone. Exposure of epithelial cells to IL-13 led to transcriptional and morphological changes in the epithelium often observed in allergic inflammation, including induction of eotaxin and loss of epithelial integrity.^{2, 4, 11, 25, 26} In this regard, our data reinforce the role of IL-13 as a molecular driver of EoE, consistent with recent findings from anti-IL-13 treatment of EoE, which affected tissue differentiation as demonstrated by normalized expression of desmoglein-1, keratin 14 and 16 and tissue-remodeling genes, such as periostin.²⁹ While IL-13 is considered critical for EoE pathogenesis, a number of other cytokines and chemokines have been implicated in the disease responses.²⁷ These molecules, such as the pro-inflammatory cytokine TNF α , likely contribute at least in part to EoE pathogenesis by altering the expression of esophageal-specific genes.²⁸

Inflammation-driven epithelial responses, such as impaired barrier function, are common for epithelia of different origin and constitute a fundamental property of allergic inflammation.^{4, 30, 31} This is supported by GWAS, which identified genetic association of epithelial cell genes including *TSLP/WDR36*, *IL33* and its receptor *ST2 (IL1RL1)* with atopic sensitization and various allergic diseases.³² GWAS identified filaggrin, a skin tissue-enriched gene, as strongly genetically associated with atopic dermatitis.^{33, 34} In the case of EoE, recent GWAS has identified genetic association with the esophagus-specific gene *CAPN14*.¹² Analysis of genes within 25 kb of variants with combined association P-value $< 1 \times 10^{-4}$ revealed two additional esophagus-specific genes—sodium channel, non-voltage gated 1 beta subunit (*SCNN1B*) and short chain dehydrogenase/reductase family 9C, member 7 (*SDR9C7*). Notably, differential expression of these genes was the part of the

signature that was sufficient to separate control from EoE cases.¹² By identifying rare mutations in 18 Eso-EoE genes by WES analysis, including *CAPN14*, we provide additional supportive biological relevance for the role of esophagus-specific genes in the pathogenesis of EoE. Furthermore, functional analysis of Eso-EoE genes, as well as mutated Eso-EoE genes, has identified protease-related activity as a putative operational pathway in disease pathogenesis. Among the genes most significantly altered in EoE were those for the serine protease inhibitors SERPINs and SPINKs, supporting a recently proposed critical role for the balance in serine protease activity in EoE pathogenesis.³⁵

An unexpected finding in our study was delineating IL-1 family members as esophagus-specific gene products. IL-36 α directly activates naïve CD4 cells, inducing IL-2 secretion and thereby priming a Th1 inflammatory response.³⁶ Given the potential role of IL-36 α in the pathogenesis of inflammation,³⁷ we assessed IL-36 α protein levels in the biopsies of patients with active EoE and controls (Figure 5). We found that IL-36 α protein was significantly decreased in EoE, implicating IL-36 α in balancing Th1/Th2 responses in the disease. We have previously reported elevated levels of IL-1 α protein in the blood and decreased esophageal expression of *IL1RN* mRNA in patients with active EoE.²⁷ Additionally, decreased expression of IL-1 α and IL-1R α has been observed in active EoE biopsies.^{38, 39} Collectively, these data implicate IL-1-related genes in the pathogenesis of EoE, presumably through modulating a balance of inflammatory and allergic signals in the context of esophageal tissue.⁴⁰ A specific feature of the IL-1 family of cytokines is a requirement for N-terminal cleavage for enhanced biological activity.³⁷ It has been reported that cysteine and serine proteases, such as calpains and elastase, can directly cleave IL-1 cytokines.⁴¹ Divergent expression of SERPINs and IL-1 cytokines in EoE biopsies suggests a functional link between proteolytic activity and IL-1-related cytokine activity in disease pathogenesis. Taken together, our data implicate IL-1-related proteins in disease progression. We speculate that IL-1 family members selectively expressed in the esophagus provide homeostatic regulation of the surface stimuli encountered by esophageal mucosa.

The finding that the esophagus normally expresses IL-1 family members, as well as protease inhibitors that likely curtail inflammation, suggests a previously unappreciated role for basal innate immunity in the esophagus. We propose that the esophagus is normally equipped with these processes to respond successfully to its robust exposure to a variety of ingested foreign substances (e.g. food). An imbalance of esophageal homeostatic processes can contribute to the development of various diseases of the upper gastrointestinal tract and perhaps lead to systemic antigen sensitization, especially in view of the impaired barrier function that develops when epithelial differentiation is lost.^{4, 6} Our findings expand our understanding of the propagation of allergic inflammation on the level of tissue molecular identity and suggest that genetic profiling and assessing expression levels of esophagus-specific genes may have diagnostic and prognostic value. We propose that the altered esophageal transcriptome driven by organ-specific imbalanced protease activity leads to profound loss of esophageal differentiation, which constitutes an integral process in allergic inflammation specific to the esophagus (Figure 6).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding support: This work was supported by National Institutes of Health R37 AI045898 (M.E.R.), R01 AI124355 (M.E.R.), U19 AI070235 (M.E.R.), F30 DK109573 and T32 GM063483 (J.T.), and P30 DK078392 (Gene and Protein Expression Core)(T.W., B.A.); the Campaign Urging Research for Eosinophilic Disease (CURED)(M.E.R.); the Buckeye Foundation (M.E.R.); the Sunshine Charitable Foundation and its supporters, Denise A. Bunning and David G. Bunning (M.E.R.); an APFED HOPE award (T.W.); and a Digestive Health Center Pilot/Feasibility award, Cincinnati Children's Hospital Medical Center (through an NIDDK P30 DK078392 mechanism) (T.W.).

We thank Shawna Hottinger for editorial assistance.

Abbreviations used

EoE	eosinophilic esophagitis
EG	eosinophilic gastritis
ALI	air-liquid interface
WES	whole-exome sequencing
FPKM	fragments per kilobase per million reads
GWAS	genome-wide association study
hpf	high-power field
EDC	epidermal differentiation complex
DSG1	Desmoglein-1
SERPIN	serine peptidase inhibitors
SPINK	serine protease inhibitor Kazal-type
GO analysis	gene ontology analysis
CAPN14	calpain 14
TGM	transglutaminase
IVL	involucrin
KRT	keratin
CRNN	cornulin
SPRR	small proline-rich protein

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Key messages

- Esophagus-specific genes are enriched in IL-1 family members and a series of proteases and protease inhibitors.
- Esophagus-specific transcripts have altered expression in EoE, with nearly all (~90%) being downregulated.
- EoE involves a loss of tissue differentiation (e.g. loss of tissue identity).
- Organ-specific, imbalanced protease activity is an integral process in allergic inflammation specific to the esophagus.
- Genetic profiling and assessing expression levels of esophagus-specific genes may have diagnostic and prognostic value.

Figure 1. Altered transcription of esophagus-specific genes in EoE

In **A**, the graph shows percentage of organ-specific genes altered in the EoE transcriptome, as assessed by RNA sequencing.³ In **B**, a Venn diagram shows the overlap of genes specifically expressed in the esophagus according to the Human Protein Atlas (Esophagus) with genes differentially expressed in the esophageal tissue of patients with active EoE compared to control esophageal tissue (EoE). For each group of genes (see text and Materials and Methods for the definitions) the number of upregulated (Up) and downregulated (Down) genes is shown (not to scale). A set of 117 overlapping genes is designated as Eso-EoE genes. In **C**, normalized expression levels for each gene (fragments per kilobase per million reads, FPKM) in esophageal biopsy tissue from patients with active EoE (EoE, blue rectangles, $n = 10$) and controls (Ctrl, green rectangles, $n = 6$) for the 12 genes most highly expressed in the homeostatic esophagus are presented as a box-and-whiskers plot. Genes upregulated in active EoE samples are in orange; the p-value for each gene is shown on the right. Statistical significance was determined using the Holm-Sidak method. In **D**, the Venn diagram shows the overlap of genes specifically expressed in the stomach according to the Human Protein Atlas with genes differentially expressed in the biopsies of patients with eosinophilic gastritis.¹⁵

Figure 2. Functional enrichment analysis of esophagus-specific Eso-EoE genes altered in EoE
 In A–D, shown are the 10 most significant terms identified for Eso-EoE genes by functional enrichment analysis in the following categories: Molecular Function (A), Biological Processes (B), Human Phenotype (C) and Protein Domain (D). Genes for the selected terms (grey bars) are listed with the corresponding fold change in expression for active EoE (EoE) compared to control (Ctrl) samples. Upregulated genes are in orange. The x axes represent the negative log (10) p-value.

Figure 3. Effect of differentiation on expression of esophagus-specific Eso-EoE genes

In **A**, schematic diagram for generating ALI cultures from EPC2 esophageal epithelial cells is shown. The cells are initially grown in submerged culture in low and then high calcium to promote initial differentiation; terminal differentiation is achieved by growing cells in ALI. Note that the presence of IL-13 during the differentiation process weakens esophageal epithelial integrity, as represented by increased spaces between the layers of cells. In **B**, Venn diagram shows overlap between Eso-EoE genes and transcriptional changes during initial differentiation. A heat map represents the $\log_2$ fold change in gene expression comparing EoE to normal biopsies and comparing d8 to d3 (Initial Diff). The analysis is focused on the 90 common Eso-EoE genes. Clustering was performed using Euclidean distance and average linkage parameters, yellow and blue colors correspond to increased or decreased expression, respectively, and the black indicates a non-significant change compared to controls.

Figure 4. Effect of IL-13 treatment on expression of esophagus-specific Eso-EoE genes in ALI
 In **A**, Venn diagram shows overlap of Eso-EoE genes with transcriptional signature of terminally differentiated ALI in the absence or presence of IL-13. D0 represents the first day and D6 represents the last day of terminal differentiation at ALI (see Figure 3 for details). Group 1 shows 32 genes altered in biopsies and in ALI in the presence and absence of IL-13. Group 2 shows 8 genes whose expression was altered in biopsies and in the presence of IL-13 during ALI (D6 vs. D6 + IL-13). Group 3 shows 28 genes whose expression was altered in biopsies and by ALI differentiation but was not affected by IL-13 (D6 vs. D0). In **B**, the heat map shows a log (2) fold change in expression of genes from IL1 and SERPINB gene families in EoE biopsies and ALI culture at the indicated conditions. Orange font represents increased expression of SERPIN genes in EoE biopsies. For **A**, **B**, heat maps show the log (2) fold change in expression of genes from indicated groups. For all heat maps, the EoE biopsy represents the expression level of the indicated genes in the EoE transcriptome compared to unaffected individuals. Clustering was performed using Euclidean distance and average linkage parameters. The yellow and blue colors correspond

to increased or decreased expression, respectively, and the black color indicates a non-significant change compared to controls. In C, expression level of indicated Eso-EoE genes following EoE diagnostic panel analysis²² was assessed by RT-PCR in control (Ctrl), active EoE (EoE) and GERD patients. Each dot represents an individual biopsy, expression was normalized to GAPDH. Error bars represent the SEM of the mean relative expression. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one way ANOVA.

Figure 5. Protein expression, protease activity and expression of differentiation markers in the esophageal biopsies of patients with EoE

In **A**, shown is a representative Western blot for the indicated proteins in control (Ctrl) and active EoE (EoE) samples ($n = 3$ biopsies). Red and green arrows point to the bands corresponding to analyzed proteins; molecular weight markers are shown on the right. In **B**, graphs show relative expression of the indicated proteins normalized to the loading control (HSP90) by Western blot, $n = 5-10$ samples for each group. Error bars represent the SEM of the mean relative expression. * $p < 0.05$, ** $p < 0.01$, Mann-Whitney test. In **C-F**, immunofluorescence of distal esophageal biopsies from control (Ctrl) patients [top row] or patients with active EoE (EoE Inflamed) [middle row] or proximal esophageal biopsies from regions that do not have active inflammation from patients with active EoE (EoE Non-inflamed) [bottom row]. Staining with the nuclear dye DAPI or with an antibody directed against the indicated differentiation marker (KRT5, KRT14, KRT4, or CRNN; KRT – keratin; CRNN – cornulin) are indicated by blue or green, respectively. White broken line indicates basal membrane; scale bar is 100 μm .

Figure 6. The model of esophagus-specific allergic inflammation

Under healthy conditions, the esophagus expresses tissue-specific markers of terminal epithelial differentiation (represented by *KRT4* and *CRNN*). Profound loss of esophageal differentiation, acquired or genetically inherited, (represented by loss of *KRT5*, *KRT14*) predisposes and/or propagates EoE. Loss of differentiation is triggered by imbalanced proteolytic activity in the esophagus, as indicated by ratio between proteases and protease inhibitors (note that protease and protease inhibitors can be either upregulated or downregulated in EoE).

Table 1

List of Eso-EoE genes with mutations identified by WES in 33 unrelated patients with EoE

Gene	Chr	Position	Reference allele	Alternate Allele	Minor allele freq (ExaC)	Mutation type	Ref Amino Acid	Alt Amino Acid	# EoE Cases w/variant
GABRP	5	170215647	G	A	0.00306	Nonsyn	V	M	3
		170224475	C	T	0.00012	Nonsyn	T	M	1
SERPINB3	18	61322980	C	T	0.00657	Nonsyn	E	K	2
		61326645	A	-	0.00014	Frameshift Del			1
		61326659	C	T	0.00009	Nonsyn	G	R	1
SPINK5	5	147475388	C	T	0.00652	Nonsyn	R	C	2
		147494001	G	A	0.00255	Nonsyn	G	D	1
		147498551	A	G	0.00314	Nonsyn	E	G	1
		17588689	T	A	0.00378	Nonsyn	L	H	1
PAD13	1	17597423	C	T	0.00666	Nonsyn	A	V	1
		17601263	T	C	0.00029	Nonsyn	I	T	1
A2ML1	12	9000164	C	G	0.00005	Nonsyn	P	R	1
		9008113	G	A	0.00005	Nonsyn	A	T	1
		9010579	C	G	0.00002	Nonsyn	Q	E	1
KRT6B	12	52841659	G	A	0.00007	Nonsyn	R	W	1
		52841685	A	G	0.00002	Nonsyn	L	P	1
		52844244	C	T	0.00002	Nonsyn	R	H	1
		55273652	T	G	0.00103	Nonsyn	S	A	1
Clorf177	1	55307347	T	G	0.00131	Stoploss	X	E	1
		150482145	G	A	0.00749	Nonsyn	A	T	1
ECM1	1	150485285	G	C	0.00002	Nonsyn	E	D	1
		31414830	G	A	0.00513	Nonsyn	L	F	1
CAPN14	2	31425971	T	G	0.00077	Nonsyn	K	T	1
		71058270	C	G	0.00329	Nonsyn	A	P	1
CD207	2	71062864	C	T	novel	Nonsyn	D	N	1
		58852298	G	C	0.00318	Nonsyn	Q	E	1
C3orf67	3	58853564	C	T	novel	Nonsyn	E	K	1

Gene	Chr	Position	Reference allele	Alternate Allele	Minor allele freq (ExaC)	Mutation type	Ref Amino Acid	Alt Amino Acid	# EoE Cases w/variant
SCEL	13	78146273	C	T	0.00039	Nonsyn	P	L	1
		78146303	C	T	0.00231	Nonsyn	S	L	1
FUT6	19	5831608	G	A	0.00386	Nonsyn	T	M	1
		5831768	C	T	0.00007	Nonsyn	V	M	1
ACER1	19	6306810	G	A	0.00002	Nonsyn	T	I	1
		6312165	C	-	0.00002	Frameshift Del			1
IGFL1	19	46733560	T	G	0.00536	Nonsyn	C	G	1
		46733750	C	T	0.00036	Nonsyn	T	I	1
PLA2G3	22	31534280	G	A	0.00549	Nonsyn	A	V	1
		31536135	-	T	0.00404	Frameshift Ins			1
CPA4	7	129948221	G	A	0.00235	Stopgain	W	X	1
DSC2	18	28648000	-	TC	0.00833	Frameshift Ins			1

Nonsyn, non synonymous mutation; Ins, insertion; Del, deletion; Chr, chromosome; freq, frequency