Broad transcriptional response of the human esophageal epithelium to proton pump inhibitors

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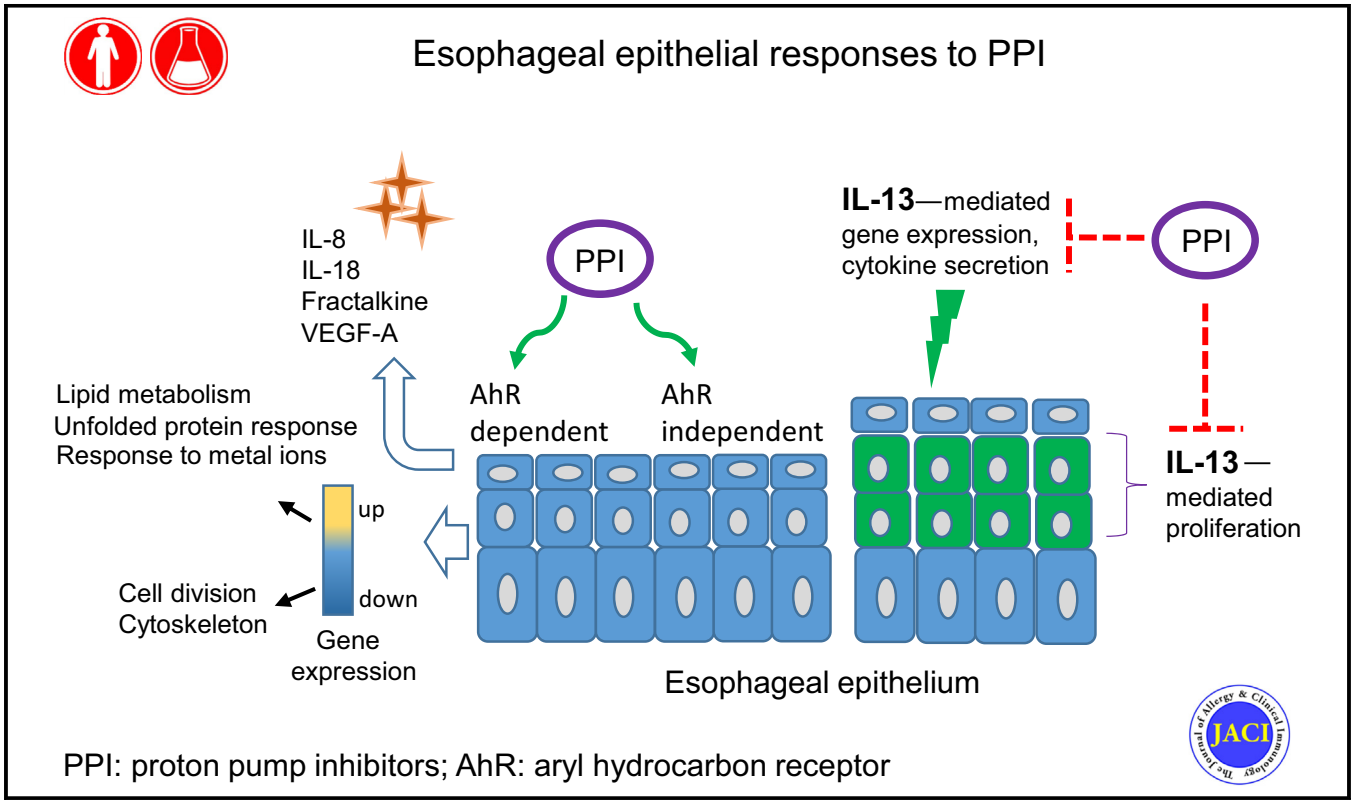
GRAPHICAL ABSTRACT



Background: Proton pump inhibitors (PPIs) have been recognized as a primary treatment of eosinophilic esophagitis (EoE), an allergic inflammatory disease of the esophageal mucosa. The mechanisms underlying esophageal epithelial responses to PPIs remain poorly understood.

Objective: We hypothesized that PPIs can counteract IL-13–mediated esophageal epithelial responses that are germane for EoE pathogenesis.

Methods: Transcriptional responses of human esophageal cells to IL-13 and the PPIs omeprazole and esomeprazole were assessed by RT-PCR and RNA sequencing. Cytokine secretion was measured by multiplex analysis and ELISA.

Results: Human esophageal epithelial cells robustly responded to PPI stimulation by inducing a set of 479 core genes common between omeprazole and esomeprazole treatments. The transcriptional response to PPIs was partially mediated

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through the aryl hydrocarbon receptor signaling pathway, as the aryl hydrocarbon receptor antagonist GNF-351 modified approximately 200 genes, particularly those enriched in metabolic processes and regulation of cell death. PPI treatment reversed approximately 20% of the IL-13 transcriptome. Functional analysis of the PPI-responsive, upregulated genes revealed enrichment in metabolic and oxidation processes, and the unfolded protein response. In contrast, downregulated genes were overrepresented in functional terms related to cell division and cytoskeletal organization, which were also enriched for the genes in the EoE transcriptome reversed by PPIs. Furthermore, PPI treatment decreased the IL-13–induced proliferative response of esophageal epithelial cells.

Conclusions: These results demonstrate broad effects of PPIs on esophageal epithelium, including their ability to curtail transcriptomic processes involved in cellular proliferation and IL-13–induced responses, and they highlight the importance of AHR signaling in mediating these responses. (J Allergy Clin Immunol 2020;■■■:■■■-■■■.)

Key words: *Proton pump inhibitors, omeprazole, esomeprazole, eosinophilic esophagitis, epithelium, IL-13, aryl hydrocarbon receptor*

Proton pump inhibitors (PPIs) such as omeprazole and esomeprazole are membrane-permeable, weak bases that covalently bind to cysteine residues of gastric H^+/K^+–adenosine triphosphatase (ATPase), leading to its inactivation and the subsequent inhibition of acid secretion from parietal cells. As acid suppressants, PPIs have historically been used for the treatment of acid-related diseases, mainly gastroesophageal reflux disease, dyspepsia, and peptic ulcer disease.[1] At the same time, the anti-inflammatory activities of PPIs have been attributed to mechanisms largely independent of their acid-suppressive effect on parietal cells and instead been driven by a direct effect of PPIs on epithelial, endothelial, and immune cells, including mast cells.[2-4]

PPIs are used extensively for treatment of eosinophilic esophagitis (EoE), a chronic allergic inflammation of the esophagus characterized by eosinophilic infiltration in the esophageal mucosa. Though initially used to distinguish gastroesophageal reflux disease from EoE, PPIs recently have become a first-line therapy for EoE, as 10% to 50% of cases respond to this single treatment. Nevertheless, the mechanism remains largely unclear.[5,6] EoE pathogenesis is driven by a transcriptional response of the esophageal tissue to the proatopic cytokine IL-13, which is largely dependent on the signal transducer and activator of transcription 6 (STAT6) transcription factor. Accordingly, blocking antibodies against IL-13, as well as inhibition of the STAT6 signaling pathway, result in decreased expression of IL-13–dependent genes and subsequent reduction of EoE responses in preclinical and early clinical studies.[7,8] The beneficial effects of PPIs in EoE have been mainly attributed to their ability to block expression and secretion of the key eosinophil chemoattractant eotaxin-3 from epithelial cells by inhibiting STAT6 phosphorylation and its binding to the eotaxin-3 promoter. Although this ability is interesting, it seems implausible that it alone can account for the broad effects of PPIs in EoE, including restoration of epithelial barrier function and differentiation.[9-12]

Abbreviations used
AHR: Aryl hydrocarbon receptor
ALI: Air-liquid interface
ATPase: Adenosine triphosphatase
CCHMC: Cincinnati Children's Hospital Medical Center
EoE: Eosinophilic esophagitis
GO: Gene ontology
KSFM: Keratinocyte serum-free medium
PPI: Proton pump inhibitor
STAT6: Signal transducer and activator of transcription 6
TEER: Transepithelial electrical resistance

Collectively, these findings highlight the critical need for elucidating the molecular mechanisms that govern esophageal epithelial responses to PPI in allergic inflammation and specifically in EoE. Herein, we have tested the hypothesis that PPIs counteract IL-13–mediated esophageal epithelial responses that are germane for EoE pathogenesis. By performing gene expression analysis, we show that omeprazole and esomeprazole have broader effects on the esophageal epithelium than previously appreciated and their effects were mainly elicited on pathways not attributed to IL-13. We have provided evidence that these effects are mediated through the aryl hydrocarbon receptor (AHR) signaling pathway, at least in part. Collectively, our results suggest that the beneficial effect of PPIs in the treatment of EoE is likely driven by partial reversal of the IL-13–mediated, disease-associated esophageal transcriptome and by IL-13–independent effects on cellular proliferation and metabolism that are largely AHR dependent.

METHODS

Cell culture and treatment

The esophageal hTERT-immortalized human epithelial cell EPC2 line was a kind gift from Dr Anil Rustgi (University of Pennsylvania). Primary human esophageal epithelial cells and fibroblasts were isolated essentially as described.[13] Briefly, esophageal biopsy specimens were mechanically dispersed and treated with collagenase and dispase following treatment with trypsin/EDTA. Cells were plated on the feeder layer of irradiated NIH 3T3 cells in keratinocyte serum-free media (KSFM) (Life Technologies, Carlsbad, Calif). Primary esophageal fibroblasts, which occasionally appeared in the culture, were separated from the epithelial cells by differential trypsinization and transferred to Dulbecco modified Eagle medium supplemented with 10% FCS medium. Clinical characteristics of the patients whose biopsy specimens were used for generating primary cells for the study are summarized in Table I in this article. For air-liquid interface (ALI) culture, 150×10^5 cells were seeded and grown to confluence while fully submerged in low-calcium (0.09 mM $CaCl_2$) KSFM on 0.4-mm pore size permeable supports (Corning Inc, Corning, NY). Confluent monolayers were then switched to high-calcium (1.8 mM $CaCl_2$) KSFM for an additional 5 days. To induce epithelial differentiation, the culture medium was removed from the inner chamber of the permeable support to expose the cells to the ALI. Barrier formation was assessed by measuring transepithelial electrical resistance (TEER) with an EVOM epithelial voltohmmeter with "chopstick" electrodes (World Precision Instruments, Sarasota, Fla). For the monolayer cultures, epithelial cells were seeded at a density of 2.5×10^5 cells/well in KSFM in a 24-well plate. Fibroblasts were seeded at 2×10^5 cells/well in a 48-well plate in 350 μL of Dulbecco modified Eagle medium supplemented with 10% FCS. The next day, the medium was replenished. After 24 hours, stimulants were added in a total of 350 to 500 μL of fresh medium for 24 hours. IL-13 (Peprotech, Rocky Hill, NJ, catalog no. 200-13) was added to a final concentration of 100 ng/mL unless

TABLE I. Primary cells used in the study

Culture No.	Primary cells	PPI-confirmed EoE	History of PPI	Peak distal eosinophils	Distal esophagus pathology
1	Epithelial	Yes	Yes	16	EoE
2		No	Yes	26	EoE
3		ND	Yes	0	Normal
4		Yes	Yes	32	EoE
5		No	ND	27	EoE
6		Yes	Yes	0	Normal
7		ND	Yes	20	EoE
8		ND	Yes	9	Abnormal
9		Yes	Yes	12	Abnormal
1	Fibroblasts	ND	Yes	0	Normal
2		ND	Yes	25	EoE

ND, Not determined.

otherwise indicated. Omeprazole (TOCRIS, Bristol, UK, catalog no. 2583) and esomeprazole (Sigma, St Louis, Mo, catalog no. E7906) were dissolved in DMSO to the stock concentration of 100 mM, aliquoted into 10-μL amounts, and stored at –80°C. PPIs were thawed once and used at a working concentration of 100 μM; cells were pretreated with the PPIs for 1 hour before adding IL-13 to the medium. GNF-351 (MedChemExpress, Monmouth Junction, NJ, catalog no. HY-102023) was dissolved in DMSO to a 20 mM stock concentration. The stock was aliquoted in 10-μL doses and stored at –80°C. Cells were pretreated with GNF-351 at a final concentration of 2 μM for 30 minutes before adding PPIs. Ki67 immunohistochemistry was performed by the Pathology Research Core at Cincinnati Children's Hospital Medical Center (CCHMC, Cincinnati, Ohio) by using CONFIRM anti-Ki67 (30-9) rabbit mAb (Roche, 790-4286).

RNA extraction and cDNA synthesis

Cells were lysed in Tripure Isolation Reagent at 700 μL/well (Sigma, catalog no. 11667165001) and stored at –20°C before RNA isolation. Chloroform was added at a volume of 140 μL per sample, and the tubes were shaken vigorously for 15 seconds and allowed to stand at room temperature for 2 to 15 minutes. Samples were centrifuged at 12,000 *g* for 15 minutes at 4°C, after which 350 μL of the upper aqueous phase was collected and mixed at a 1:1 ratio with 100% ethanol. Further isolation was performed by using the Quick-RNA MicroPrep Kit (Zymo Research, Irvine, Calif, catalog no. R1051) per the manufacturer's instructions. The RNA concentration was measured by Nanodrop, and the RNA integrity number was determined by the Gene Expression Core at CCHMC by using the Agilent 2100 Bioanalyzer. cDNA synthesis was performed according to the protocol of the ProtoScript II Reverse Transcriptase kit (NEB, Ipswich, Mass, catalog no. M0368).

ELISA and multiplex analysis

Cells were treated with IL-13 at 100 ng/mL for 24 hours. Before collection of the supernatants, NaCl was added to each well at a final concentration of 500 mM and the plate was rotated for 15 to 30 minutes at room temperature. Supernatants were centrifuged at 12,000 *g* at 4°C for 15 minutes before use. ELISA was performed with the kits for human CCL26/Eotaxin-3 (R&D Systems, Minneapolis, Minn, catalog no. DY346) per the manufacturer's instructions. Multiplex analysis was performed by Eve Technologies (Calgary, Canada) using the Human Cytokine Array/Chemokine Array 65-Plex Panel (HD65). Experiments were performed with 1 or 2 independent cultures of EPC2 cells and 2 primary esophageal epithelial cells in duplicates. Only cytokines detected at a concentration higher than 5 pg/mL for at least 1 treatment were used for the analysis. A *t* test was performed to assess significant changes in the secretion by comparing individual treatments (IL-13, omeprazole) with the untreated cells and combined treatment (IL-13 plus omeprazole) to IL-13 alone. *P* values less than .05 were considered significant.

3′ RNA sequencing and data processing

A submerged monolayer culture of the EPC2 cells seeded at density of 2.5×10^5 cells/well in a 24-well plate was used for the RNA sequencing. RNA sequencing was performed with high-quality RNA (RNA integrity number > 8) by using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen, Vienna, Austria, catalog no 015.96). Libraries were subjected to quality control and concentration measurements at the Gene Expression Core at CCHMC. Libraries were diluted to final concentrations of 5 nM and sequenced on a HiSeq 4000 Illumina sequencing machine at the Genomics and Cell Characterization Core Facility at the University of Oregon with 100- to 150-bp-length single reads. Data analysis and visualization were performed by using the CLC Genomics Workbench, version 12.0.3 (Qiagen, Hilden, Germany). Briefly, raw data sequencing files were imported into the software, sequences were trimmed from the adapters, and alignment was performed by using the HG38 human genome. Total read counts for the samples were between 1.3 and 6.8 million per sample, and 85% to 95% of the reads were successfully mapped to the forward DNA strand. Of these reads, 63% to 79% were mapped to the protein-coding regions. Differentially expressed genes were defined by fold change and statistical filtering and clustered, as indicated in the figure legends. For some heat maps, Cluster 3.0 was applied for clustering genes by 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). 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 with the ToppGene suite[14] (https://toppgene.cchmc.org/). Unless otherwise indicated, differentially expressed genes were used as input for GO analysis. Venny (https://bioinfogp.cnb.csic.es/tools/venny/) was used to intersect gene lists. As indicated, expression data were intersected with the EoE transcriptome, and the list of 1607 significantly dysregulated transcripts was identified by comparing gene expression in the biopsy specimens of 10 patients with active EoE (9 of which were irresponsive to the PPI treatment) with that in normal controls by the RNA sequencing analysis.[15]

RESULTS

Transcriptional response of submerged EPC2 cells to IL-13 and PPIs

We aimed to investigate the ability of PPIs to reverse IL-13–mediated transcriptional responses in EoE. To this end, we examined the immortalized esophageal epithelial cell line EPC2, which has been widely used to model epithelial properties of the homeostatic and diseased human esophagus.[13,16]

We analyzed global gene expression profiles by RNA sequencing of submerged monolayer culture of the EPC2 cells treated with IL-13, omeprazole, and esomeprazole either alone or in combination. Principal component analysis separated the gene expression profiles of each group (Fig 1, *A*). Collectively,

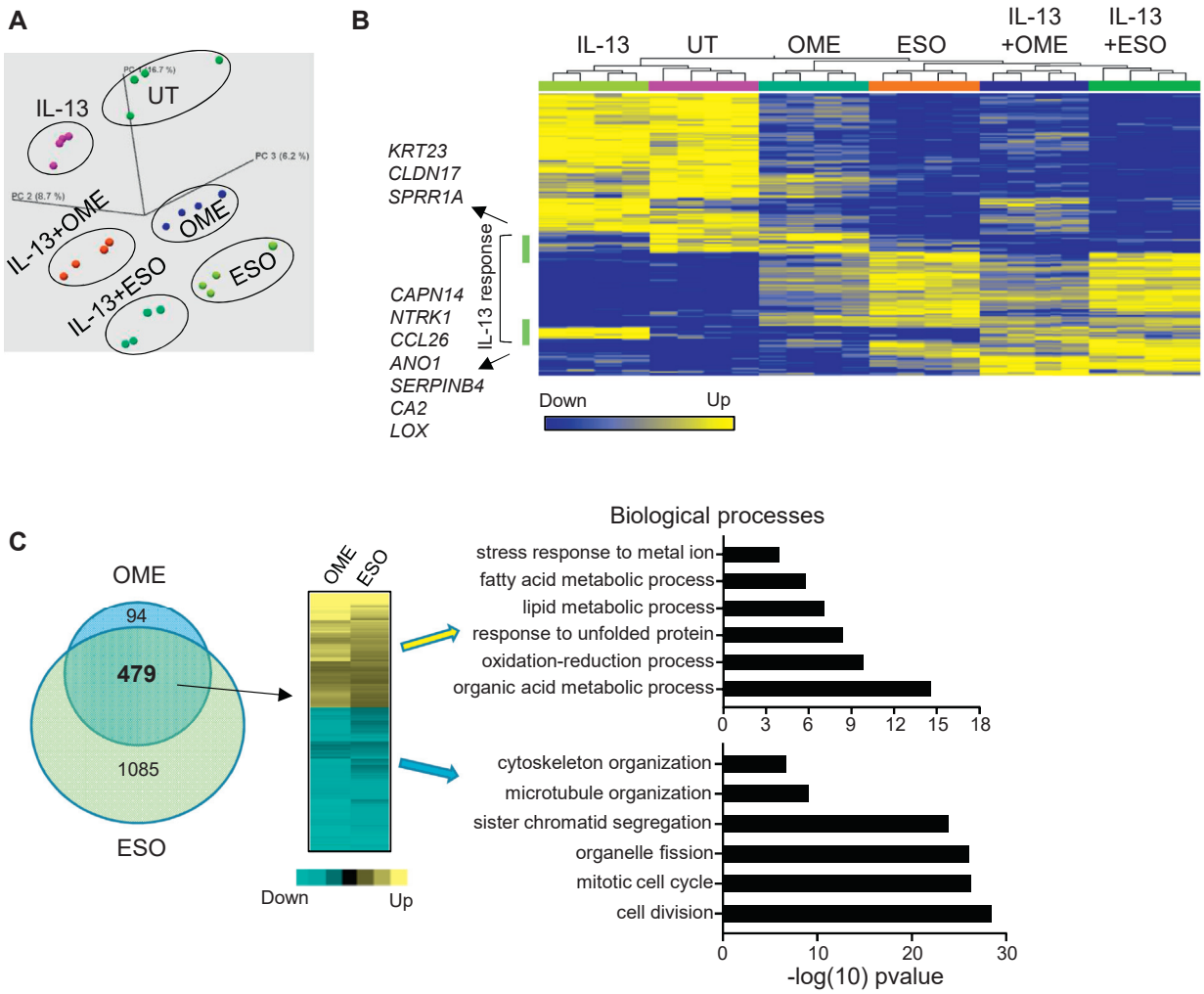

FIG 1. Transcriptional signature of EPC2 cells in response to IL-13 and PPIs. **A,** Principal component analysis of RNA sequencing results from EPC2 cells (n = 4 for each stimulation is shown). **B,** The heat map shows clustering of differentially expressed genes (ANOVA; FDR $P < .05$; fold change 4). The IL-13 response represents genes dysregulated by IL-13; examples of genes commonly associated with IL-13 signature are indicated. Note the stronger response to esomeprazole (ESO) than to omeprazole (OME), as judged by the scale of changes in the expression level of genes (compare intensities of yellow and blue colors in OME vs ESO samples). **C,** The Venn diagram shows overlap of the genes dysregulated by OME and ESO. The heat map shows the expression level change for the 479 genes common to OME and ESO compared with untreated (UT) cells. The bar graphs show biologic processes enriched for upregulated and downregulated genes defined by GO analysis (FDR $P < .05$). For (**B**) and (**C**), yellow and blue colors represent upregulated and downregulated genes, respectively.

709 differentially expressed genes were identified by comparison between treatment groups (ANOVA; $P < .05$; fold change > 4; with a Benjamini-Hochberg false discovery rate [FDR] (Fig 1, *B*). Several characteristics of the cellular response to these stimuli became apparent from this analysis. First, EPC2 cells responded to IL-13 by upregulating and downregulating a number of genes, many of which were commonly observed in IL-13–mediated responses, including those in the EoE transcriptome (see Fig E1 and Table E1 in this article's Online Repository at www.jacionline.org [see the bolded genes in the untreated vs in the IL-13–treated sheet]). Among the most highly upregulated genes were *CCL26*, *TNFAIP6*, *NTRK1*, *SERPINB4*, *CAPN14*, and *ANO1*, which are typically associated with IL-13 responsiveness of the esophageal epithelium in active EoE, as the expression of these genes is STAT6 dependent.[8,17] Second, the degree of transcriptional response was surprisingly stronger for PPIs than for IL-13, as is evidenced by a larger number of dysregulated genes in PPI–treated cells than in IL-13–treated cells (see also Fig E2 in this article's Online Repository at www.jacionline.org). Notably, despite substantial overlap in the gene expression signature following omeprazole and esomeprazole stimulation, the latter induced a stronger response, as was evident by the scale of changes in gene expression (increased intensity of yellow and blue colors). Indeed, pairwise analysis revealed that 188 genes were altered in the cells treated with IL-13, whereas 573 genes and 1564 genes were dysregulated in response to

omeprazole and esomeprazole, respectively (FDR $P < .05$; 2-fold change [see Table E1]). Overall, 479 genes were regulated by both omeprazole and esomeprazole, demonstrating remarkable similarity in their regulation (Fig 1, *C*). Functional analysis of the upregulated genes shared between PPIs revealed enrichment of GO terms associated with metabolic and oxidation processes, as well as the unfolded protein response and lipid metabolism. In contrast, the downregulated genes were overrepresented in GO categories related to cell division and cytoskeletal organization (Fig 1, *C* [biologic processes]).

Transcriptional response of submerged primary cells to IL-13 and PPIs

To further assess epithelial responses to IL-13 in the absence and presence of the PPIs, the expression of several IL-13– and PPI-responsive genes in 4 primary epithelial cell lines were examined. Similar to EPC2 cells, primary cells robustly respond to PPIs, by increasing expression of *CYP1A1*, *HMOX1*, and *MT1H,* with a stronger response following esomeprazole stimulation (Fig 2, *A*). Whereas following stimulation with IL-13 expression of several IL-13–inducible genes, including *NTRK1*, *SERPINB3,* and *TNFAIP6,* was modestly decreased by esomeprazole in combination with IL-13 compared with IL-13 alone, PPIs did not prevent robust upregulation of these genes (Fig 2, *B*).

To evaluate the cellular specificity of the PPI response, the expression of PPI-inducible genes in primary esophageal epithelial cells and primary esophageal fibroblasts was examined. Unlike epithelial cells, fibroblasts did not induce *CYP1A1* and *HMOX1*, whereas expression of *MT1B* and *MT1H* was upregulated, albeit variably (see Fig E3, *A* in this article's Online Repository at www.jacionline.org). Similar to the epithelial response, induction of the IL-13 target genes *CCL26* and periostin (*POSTN*) was not primarily affected by the PPI treatment (see Fig E3, *B*). Collectively, these results suggest cellular specificity of the transcriptional responses to PPIs.

Transcriptional response of differentiated epithelial cells to PPIs

Epithelial cells grown at the ALI represent a commonly used *in vitro* model for studying esophageal epithelial differentiation.[16] In this model, epithelial stratification and differentiation are induced following exposure of the monolayer cells grown on the membrane to the air. This results in formation of a multilayered epithelial culture characterized by increased TEER. In the presence of IL-13, cellular proliferation is increased,[18] the epithelial barrier is damaged, and TEER is decreased. We utilized this system to test the response to the PPIs in the EPC2 cell line and primary esophageal epithelial cells. Following exposure of cells to the ALI (ALI D1), the cells were treated for 3 days with IL-13, followed by cotreatment with IL-13 and PPIs for an additional 48 hours (Fig 3, *A*). As expected, IL-13 treatment substantially decreased TEER compared with in the untreated samples, and it was not reversed by the study PPIs (Fig 3, *A*), indicating that the PPIs do not reverse IL-13–mediated loss of the epithelial barrier integrity. However, epithelial cells grown at the ALI robustly responded to the PPI stimulation, as was evident by transcriptional induction of the PPI-inducible genes *CYP1A1*, *MT1B* and *MT1H* (Fig 3, *B*). Given that PPI treatment led to the downregulation of the genes related to cell proliferation (Fig 1, *C*), we assessed the proliferative potential of IL-13 in the presence and absence of the PPIs (Fig 3, *C*). During the last 48 hours of the culture, cells were treated cells with IL-13 in the presence or absence of the PPIs and proliferation was assessed by immunostaining with Ki67, a nuclear protein that is associated with cellular proliferation.[19] IL-13 increased proliferation of the basal cells in the culture,[18] whereas omeprazole and esomeprazole significantly diminished this effect (Fig 3, *C*). Collectively, these results suggest that esophageal epithelial cells respond to PPIs independent of their differentiation state and support the antiproliferative potential of the PPIs in the context of the IL-13 response.

Contribution of the AHR signaling pathway to transcriptional response to PPIs

AHR expression is elevated in the esophageal biopsy specimens of patients with EoE compared with in controls and in IL-13–treated EPC2 cells grown at the ALI compared with in untreated cultures (see Fig E4 in this article's Online Repository at www.jacionline.org).[15,16] Omeprazole and esomeprazole have been shown to exert responses through activation of AHR, leading to the strong induction of several cytochrome P450 isoenzymes, including *CYP1A1*.[20,21] Accordingly, EPC2 cells robustly increased expression of *CYP1A1* in response to omeprazole and esomeprazole (~20-fold increase), and this induction was blunted by the cell-permeable, high-affinity antagonist of AHR signaling GNF-351 (see GNF in Fig 4, *A* [*upper graph*]).[22] Notably, basal expression level of *CYP1A1* was also decreased in GNF-351–treated cells compared with in untreated cells, indicating active engagement of the AHR signaling pathway under these conditions. In contrast, induction of *MT1H* was not affected by GNF-351 (Fig 4, *A* [*lower graph*]).

We subsequently hypothesized that AHR signaling was important for PPI-mediated transcriptional responses in esophageal epithelial cells. To test this hypothesis, we pretreated cells with GNF-351 followed by stimulation with PPIs and performed RNA sequencing. Unsupervised clustering of the affected genes (ANOVA; FDR $P < .05$; 4-fold change) revealed good separation of samples based on the treatment groups (Fig 4, *B*). Comparing the range of effect of GNF-351 pretreatment on PPI stimulation, we found that GNF-351 treatment primarily affected the response to esomeprazole, as was evident by the scale of the changes in gene expression (compare intensities of yellow and blue colors in the ESO + GNF vs ESO and OME + GNF vs OME columns). As a positive control, expression of *CYP1A1* and *CYP1B1* genes was reversed by GNF-351 pretreatment in both omeprazole- and esomeprazole-treated cells. Regarding the response to esomeprazole, 187 genes were affected by GNF-351 in combination with esomeprazole versus when esomeprazole was used alone; the basal expression of these 187 genes was not altered by GNF-351 pretreatment (Fig 4, *C*). Notably, these genes were primarily upregulated by esomeprazole, and this induction was blocked by the AHR signaling antagonist GNF-351 (see Table E2 in this article's Online Repository at www.jacionline.org). GO analysis revealed that AHR signaling is overrepresented by genes associated with biologic processes related to metabolism, response to stimuli, and cell death (Fig 4, *D* and *E*). Collectively, these findings demonstrate that PPI-mediated transcriptional responses in the esophageal epithelium are partially mediated by AHR signaling.

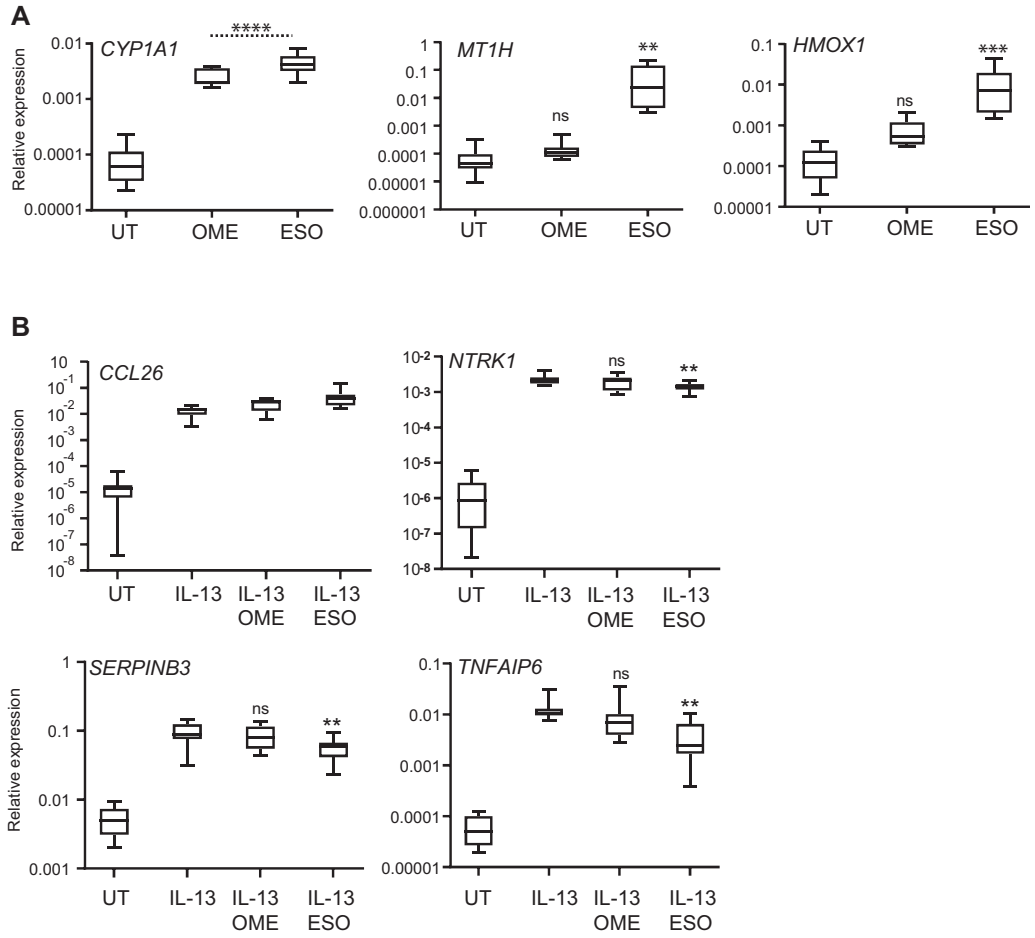

FIG 2. Transcriptional response of primary epithelial cells to IL-13 and PPIs. **A** and **B,** The expression level of the indicated genes was assessed by RT-PCR and normalized to the housekeeping gene *GAPDH*. Primary cell lines were stimulated with the PPIs alone (**A**) or in combination with IL-13 (**B**). Combined data for 4 independent primary cell lines in duplicates are shown as a box and whiskers plot, ****$P <$ .0001; ***$P <$ .001; **$P <$ .01; ANOVA with Holm-Sidak correction. For box and whisker plots, the box represents the 50th percentile of the data, the whiskers show minimum and maximum values, and the line in the box represents the median. *ESO*, Esomeprazole; *ns*, not significant; *OME*, omeprazole; *UT*, untreated.

Combined effects of IL-13 and PPIs on cytokine secretion and gene expression

Previous research has primarily focused on the ability of PPIs to decrease expression of *CCL26* (eotaxin-3) by decreasing the binding of the IL-13 signaling molecule STAT6 to the *CCL26* promoter.[10] Consistent with these observations, we observed a modest (~2-fold) decrease of the CCL26 protein level in IL-13–treated EPC2 cells and primary esophageal epithelial cells, but not in the primary esophageal fibroblasts following pretreatment with PPIs (Fig 5, *A* and see also Fig E5 in this article's Online Repository at www.jacionline.org). Notably, this effect was reversed by the AHR inhibitor GNF-351 (Fig 5, *B*).

To further assess interaction between IL-13 and PPI responses, we performed a multiplex analysis of 65 cytokines in EPC2 and primary esophageal epithelial cell stimulated with IL-13 and esomeprazole either alone or in combination. Cytokines whose average secretion exceeded a concentration of 5 pg/mL for at least 1 stimulation were included in the analysis (see Table E3 in this article's Online Repository at www.jacionline.org). We subsequently identified 12 cytokines that were significantly dysregulated in the epithelial cells in response to IL-13 and esomeprazole compared with in untreated cells, or a combination of both compared with in response to IL-13 alone (*t* test; $P < .05$). Six cytokines were shared between EPC2 and primary cells following this analysis (Fig 5, *C*). Secretion of fractalkine, IL-8, IL-18, and VEGF-A was elevated by treatment with esomeprazole alone (Fig 5, *D*). Moreover, eotaxin-3 and RANTES were upregulated by IL-13 and repressed by esomeprazole (Fig 5, *E*). These results show that the response of the epithelial cells to PPIs includes increased secretion of cytokines, such as IL-8 and IL-18, and that PPIs can modify IL-13–mediated cytokine secretion.

To further explore the relationship between PPIs and IL-13–mediated responses in the esophageal epithelium, we assessed the effect of combined stimulation with IL-13 and PPIs on gene expression. By comparing the transcriptional profile of EPC2

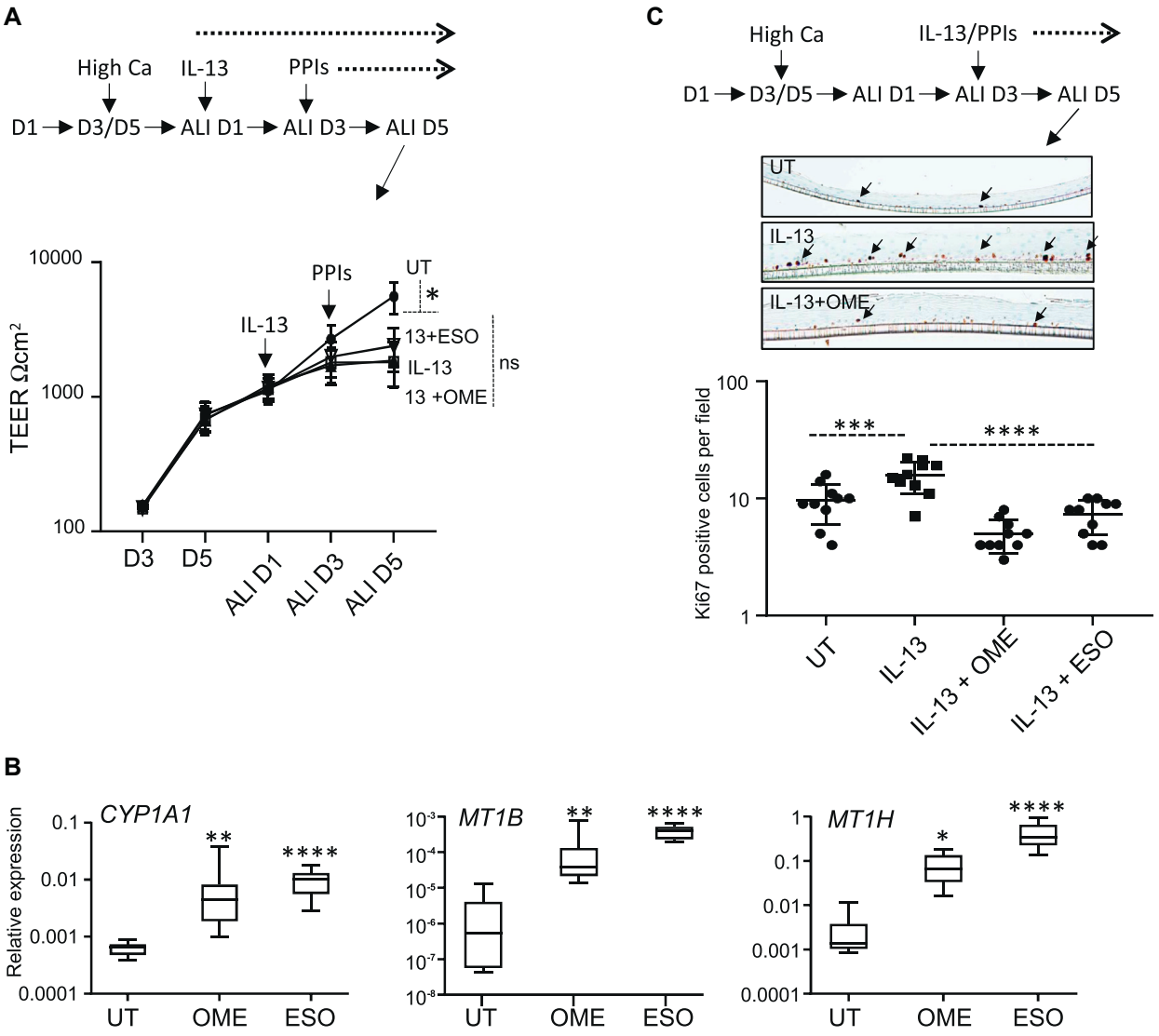

FIG 3. Response of differentiated epithelial cells to IL-13 and PPIs. **A,** Schematics above the graph outlines the experimental approach; dotted arrows indicate duration of treatment. Cultures were treated with IL-13 at 20 ng/mL and PPIs at a final concentration of 100 μM. The graph shows the TEER of the epithelial cells grown at the ALI culture. Untreated (UT) cells show the highest TEER compared with in the other cultures. **P* < .05; *t* test with Holm-Sidak correction. **B,** The expression level of the indicated genes was assessed by RT-PCR and normalized to the housekeeping gene *GAPDH*. Combined results for 4 independent primary cell lines and EPC2 cells performed in duplicates are shown as a box and whiskers plot. *****P* < .0001; ***P* < .01; **P* < .05; Kruskal-Wallis test. For box and whisker plots, the box represents the 50th percentile of the data, the whiskers show minimum and maximum values, and the line in the box represents the median. **C,** Proliferative response to IL-13 in the absence and presence of PPIs was assessed by staining of the Ki67 marker. IL-13 was used at 100 ng/mL, and cells were pretreated with the PPIs for 1 hour before stimulation with IL-13. Schematics outline of the experimental approach; representative images of the stained sections are shown above the graph. Arrows point to the brown nuclei of Ki67-positive cells. The graph shows the number of the Ki67-positive cells in 10 high-power fields for 3 cultures per condition. *****P* < .0001; ****P* < .001; ANOVA with Holm-Sidak correction. *ESO,* Esomeprazole; *ns,* not significant; *OME,* omeprazole.

cells treated with IL-13 alone or pretreated with the PPIs before IL-13 stimulation, we identified 86 genes whose expression was affected by costimulation with the PPIs and IL-13 (27 + 51 + 8 genes shared with untreated vs IL-13–treated circle in the Venn diagram; FDR *P* <05; 2-fold change [Fig 5, *F*]). Expression of 32 of these genes was reversed by both PPIs compared with by IL-13 treatment alone. This effect was more pronounced on the genes upregulated by IL-13, including those most highly upregulated in the EoE transcriptome (Fig 5, *F*). Taken together, these results demonstrate that although PPIs can partially reverse the IL-13–mediated transcriptome, the effect on gene expression in the esophageal epithelium is largely IL-13 independent.

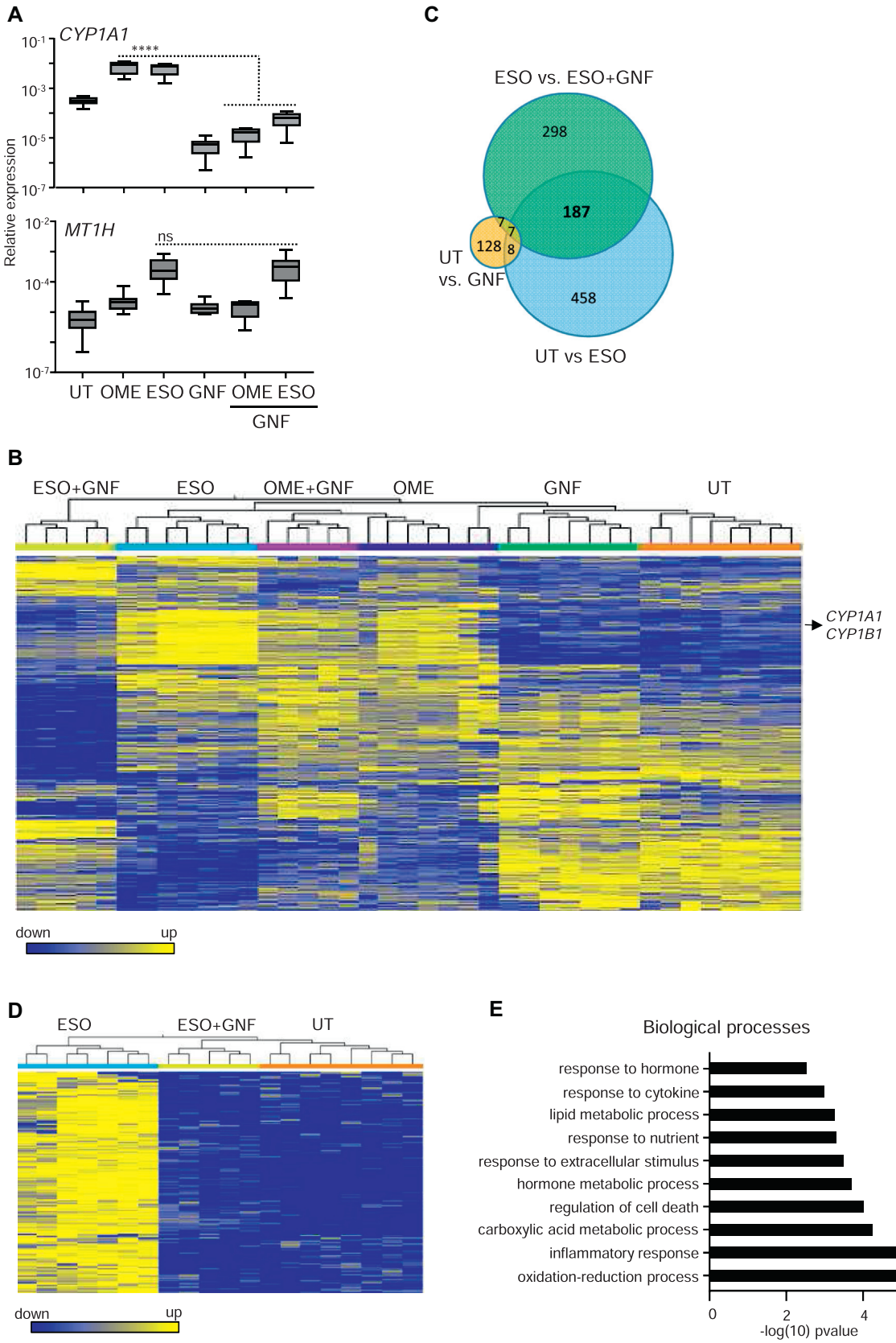

FIG 4. Contribution of the AHR signaling pathway to transcriptional response to PPIs. **A,** The expression level of *CYP1A1* and *MT1H* normalized to the housekeeping gene *GAPDH* was assessed by RT-PCR; the combined data for 4 independent experiments are shown as box and whiskers plot, ****$P < .0001$; ANOVA with Holm-Sidak correction. On the plot, the box represents the 50th percentile of the data, the whiskers show

Functional enrichment analysis of the epithelial response to PPIs

We hypothesized that the biologic processes critical for EoE pathogenesis may be inversely regulated by PPIs. To test this hypothesis, we intersected the PPI-mediated gene expression signature with the EoE transcriptome, the list of genes significantly transcriptionally dysregulated in the biopsy specimens of patients with active EoE compared with in the unaffected controls.[15] This analysis revealed 327 shared genes, and the expression of approximately 70 of those genes with PPIs changed in the direction opposite that of the EoE transcriptome (Fig 6, *A*). Notably, genes with this inverse expression were enriched for GO terms associated with the cell cycle and microtubule organization (Fig 6, *B*). Collectively, these results suggest that PPIs regulate biologic processes germane to EoE pathogenesis, including cell division and metabolism.

DISCUSSION

Given the now widely accepted beneficial effects of PPIs for the treatment of EoE, understanding the mechanisms behind their effects is a timely scientific pursuit. Using human esophageal epithelial cells, we have demonstrated robust responses to omeprazole and esomeprazole at the transcriptional level and shown that these responses are partially mediated by the AHR signaling pathway. We have shown that although IL-13–mediated gene expression and cytokine secretion are partially reversed by PPIs, the PPI-induced responses are largely separate from those induced by IL-13. This finding broadens the protective and therapeutic effects of PPIs compared with simply inhibiting the end-stage inflammatory responses triggered by IL-13. The results also suggest potential benefit of co-administering PPI and anti–type 2 (eg, anti–IL-13 and anti–IL-4Rα) therapies. We have demonstrated that the biologic pathways regulated by PPIs are primarily associated with metabolic responses and the cell cycle. Importantly, the latter are enriched for genes from the EoE transcriptome that are reversed by PPIs. A potential antiproliferative effect of the PPIs is further supported by the ability of the PPIs to decrease IL-13–mediated proliferation *in vitro*. Collectively, we have provided evidence that the esophageal epithelial responses to PPIs are broader than previously reported and that PPIs regulate biologic processes germane for EoE pathogenesis. Although limited to the submerged epithelial culture, these findings represent the first step in explaining the emerging positive effects that PPI therapy has for EoE, uncovering their broad ability to mechanistically regulate homeostatic epithelial processes (metabolism and cell proliferation), likely providing protection from inflammatory insults, particularly those driven by IL-13 in the case of EoE.

Despite their primary use for acid-related disorders, the beneficial effects of PPIs extend beyond their antisecretory properties. For example, PPIs can protect mice against TLR-dependent and independent acute systemic inflammation by inhibiting TNF-α and IL-1β production by macrophages.[23] PPIs are also capable of effectively scavenging reactive oxygen species, thereby protecting DNA from damage during oxidative stress.[24,25] Treatment with esomeprazole has been effective in preventing fibrosis in lung and ocular tissues by downregulating TGF-β, fibronectin, and matrix metalloproteases.[2,26] In addition, anti-inflammatory properties have been attributed to the ability of PPIs to block the production of IL-6, IL-8, and TNF-α.[26,27] Consistent with previous findings, PPIs efficiently counteracted IL-13–mediated secretion of eotaxin-3 in EPC2 and primary esophageal epithelial cells, but not in primary fibroblasts. Herein, we have extended this finding by suggesting that the beneficial effects of PPIs greatly extend past inhibition of eotaxin-3 alone. For example, we observed substantial inhibition of another eosinophil and T-cell chemoattractant, RANTES, the expression of which is also increased in the esophageal tissue of patients with EoE.[28] At the same time, our data show that esomeprazole increased secretion of several cytokines, including IL-18, which on the one hand has been implicated in promoting EoE[29] but on the other hand had a protective role in allergic asthma.[30] The consequences of the IL-18 induction in response to PPIs might depend on the context of the environmental cues at the time of the intervention. Although PPIs are considered beneficial for the treatment of EoE, epidemiologic studies have linked early-life exposure to PPIs as a substantial risk factor for EoE and food allergy, although the operational mechanisms are unclear.[31,32] Our findings, although limited to the epithelial responses in the cell culture, have potential implications for understanding these mechanisms. The demonstration that PPIs have broad transcriptional consequences on esophageal epithelial cells highlights their potential ability to modify processes (eg, metabolism and proliferation) that are likely germane for response to subsequent inflammatory triggers associated with the development of chronic allergic responses. Transcriptional response of other cell types involved in the propagation of the allergic inflammation, including fibroblasts and mast cells, are likely contributing to the overall clinical benefits of the PPIs.

Signaling and metabolic pathways of PPIs differ depending on their chemical structure. For example, omeprazole and esomeprazole signal through AHR, which integrates multiple environmental signals.[21] Subsequently, activation of AHR leads to induction of the family of CYP450 enzymes that in turn metabolize PPIs.[20] The increased expression of *CYP1A1* and *CYP1B1* enzymes in the EPC2 and primary esophageal epithelial cells in response to PPIs and the efficient blocking of this induction by the AHR antagonist GNF-351 indeed indicate that the omeprazole- and esomeprazole-induced response is

minimum and maximum values, and the line in the box represents the median. **B,** The heat map shows clustering of differentially expressed genes (ANOVA; FDR $P < .05$, fold change 4). Note the stronger inhibitory effect of GNF-351 (GNF) on esomeprazole (ESO) than omeprazole (OME) (compare the intensities of the yellow and blue colors of OME vs OME-GNF and ESO vs. ESO-GNF). **C,** The Venn diagram shows the overlap of genes dysregulated by the indicated stimuli ($P < .05$; 1.5-fold change). **D,** The heat map shows clustering of 187 common genes from (**C**). For (**B**) and (**D**), the yellow and blue colors represent upregulated and downregulated genes, respectively. **E,** Shown are the representative biologic processes significantly enriched for the 187 common genes from (**C**) defined by GO analysis (FDR $P < .05$). *ns,* Not significant; *UT,* untreated.

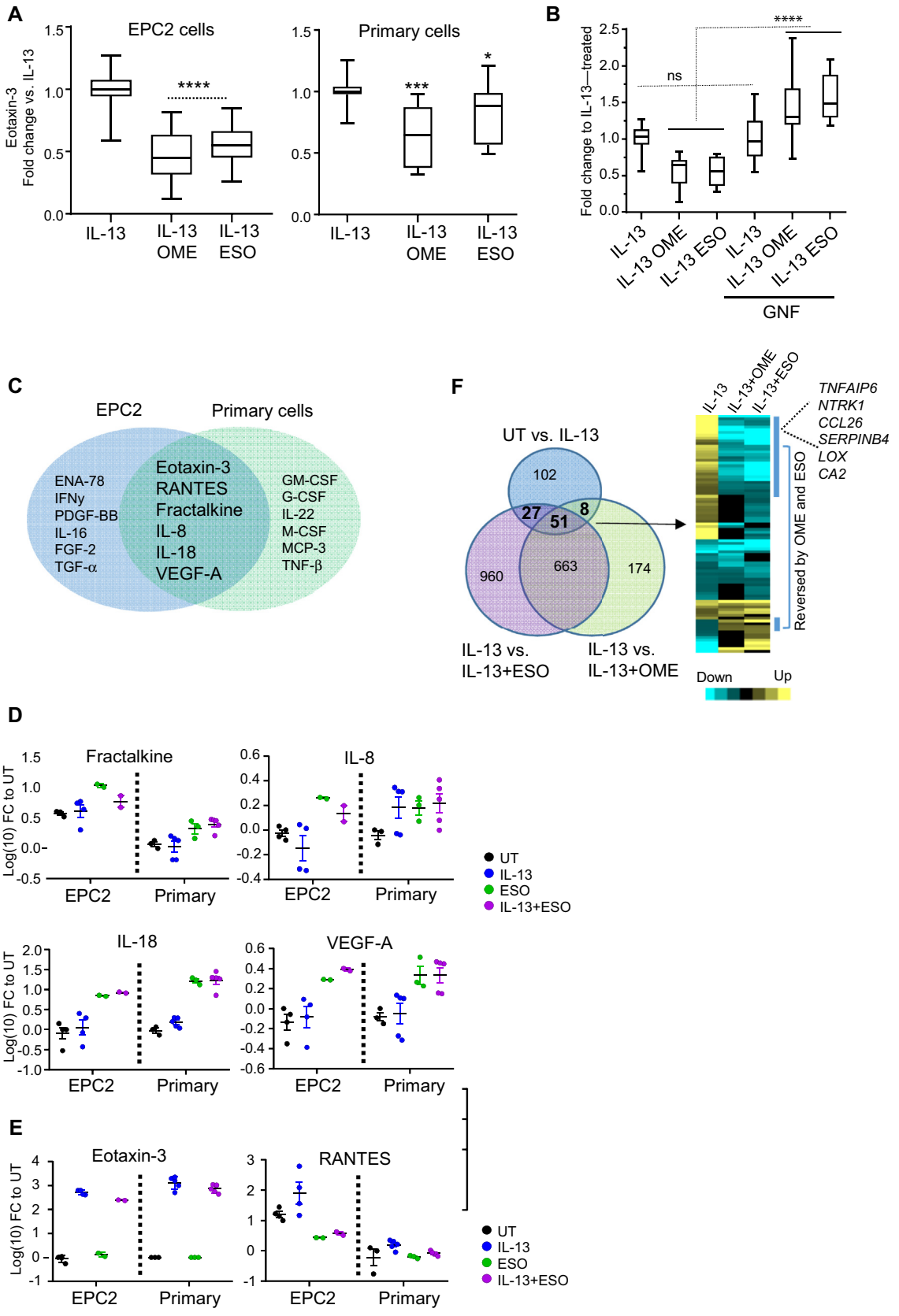

FIG 5. Effect of PPIs on IL-13–mediated transcription. **A,** The relative secretion level of eotaxin-3 in EPC2 and primary esophageal epithelial cells is shown as box and whiskers plot and represents the combined data for n = 24 (EPC2 cells) and n = 11 (primary cells) independent experiments, ****$P < .0001$; ***$P < .001$; *$P < .05$; ANOVA with Holm-Sidak correction. On the plot, the box represents the 50th percentile of the data, the

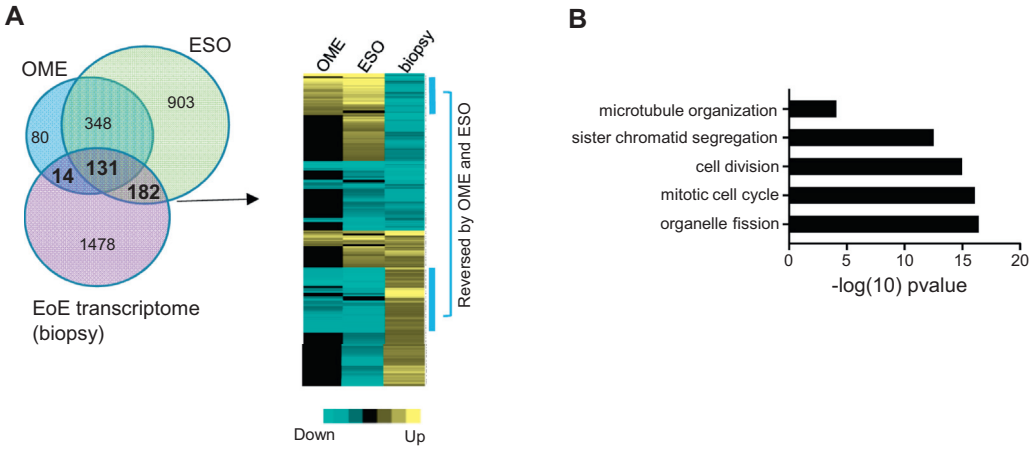

FIG 6. Functional enrichment analysis of transcriptional response to PPIs. **A,** The Venn diagram shows the intersection of genes dysregulated by PPIs and the EoE transcriptome.[15] The shared genes (*bolded*) were used to generate a gene expression heat map using log2 fold change compared with either untreated (UT) cells (for omeprazole [OME] and esomeprazole [ESO]) or control patients (for EoE transcriptome). Genes whose expression with PPIs changed in the direction opposite that of the EoE transcriptome are indicated by lines. **B,** The bar graph shows the biologic processes enriched for genes oppositely affected by PPIs (vs EoE transcriptome) as defined by GO analysis (FDR $P < .05$). Yellow and blue colors represent upregulated and downregulated genes, respectively.

mediated by the AHR signaling pathway. Whether AHR-dependent responses are operational in the patient's response to PPIs remains to be investigated; however, the increased expression of the AHR in the biopsy specimens of patients with active EoE compared with in the controls supports the relevance of this mechanism. In contrast to omeprazole and esomeprazole, the PPI rabeprazol is neither a ligand for AHR nor a primary target of CYP450 enzymes.[21] This suggests that mechanisms other than AHR signaling are likely contributing to the effects of PPIs. For example, blocking of eotaxin-3 secretion in IL-13–stimulated human sinonasal epithelial cells by PPIs is linked to inhibition of the nongastric H^+/K^+ ATPase *ATP12A*.[33] In mast cells, omeprazole likely blocks a vacuolar-type H^+ ATPase (V-ATPase),[4] whereas in melanocytes, omeprazole reduces melanogenesis by inhibiting a copper-transporting P-type ATPase.[34] Our data show that the AHR pathway partially contributes to the transcriptional response to PPIs, although the contribution of AHR and other signaling pathways to the beneficial effects of the PPIs in EoE, including on the EoE transcriptome, warrants further investigation.

Basal zone hyperplasia is a major histopathologic characteristic of EoE.[35] Although the molecular mechanisms behind basal zone hyperplasia are not fully understood, IL-13 signaling is likely involved.[18,36] Our finding that PPIs can inversely regulate genes related to cell cycle in the EoE transcriptome and counteract IL-13–mediated proliferation in culture expands PPI responses in EoE beyond simply regulating eosinophilic infiltration.[6] Moreover, the contribution of other biologic processes directly regulated by PPIs, such as lipid metabolism and unfolded protein responses, are likely critical for understanding the beneficial role of PPIs in the treatment of EoE. Notably, metallothionein transcripts, which are some of the most highly upregulated genes induced by PPIs, encode for proteins that have immunomodulatory activities.[37] In summary, our results demonstrate that the esophageal epithelium is a critical target for PPIs in EoE and that PPIs regulate biologic processes germane to EoE pathogenesis, including cell proliferation and metabolism. These findings call attention to further understanding the pathways that are regulated by PPIs, as they likely underlie those involved in EoE pathogenesis, including AHR signaling.

whiskers show minimum and maximum values, and the line in the box represents the median. **B,** The relative secretion of eotaxin-3 was assessed by ELISA in EPC2 cells treated with IL-13 and PPIs in the presence of GNF-35, as indicated. Secretion was normalized to IL-13 treatment; combined data for 7 independent experiments are shown. ****$P < .0001$; ANOVA with Holm-Sidak correction. For box and whisker plots, the box represents the 50th percentile of the data, the whiskers show minimum and maximum values, and the line in the box represents the median. **C,** Cytokines produced by EPC2 and primary esophageal epithelial cells in response to IL-13 and/or esomeprazole (ESO) were assessed by the multiplex array. Cytokines that were secreted at a concentration of of more than 5 pg/mL and significantly changed in at least 1 of the stimulations (IL-13, ESO vs untreated [UT], IL-13 + ESO vs IL-13; $P < .05$ [see Table E3]) are indicated. **D** and **E,** Expression of the cytokines shared between EPC2 and primary cells is shown as log10 fold change (FC) relative to UT cells (average ± SEM). Each dot represents an independent sample. **F,** The Venn diagram shows the intersection of genes dysregulated by either IL-13 alone or in combination with PPIs. The heat map shows the expression of the 86 shared genes (*bolded in the Venn diagram*). The genes whose expression is reversed by PPIs are indicated by lines; examples of IL-13–upregulated genes reversed by PPIs are shown. Yellow and blue colors represent upregulated and downregulated genes, respectively. *OME*, Omeprazole.

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Clinical implications: This work contributes to the understanding of the beneficial effects of PPI treatment of patients with EoE, including the ability of PPI to curtail transcriptomic processes involved in cellular proliferation and to a lesser extent IL-13–induced responses. The results suggest the potential advantage of co-administering PPI and anti–type 2 (eg, anti–IL-13 and anti–IL-4Rα) therapies and the role of AHR signaling in EoE.

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