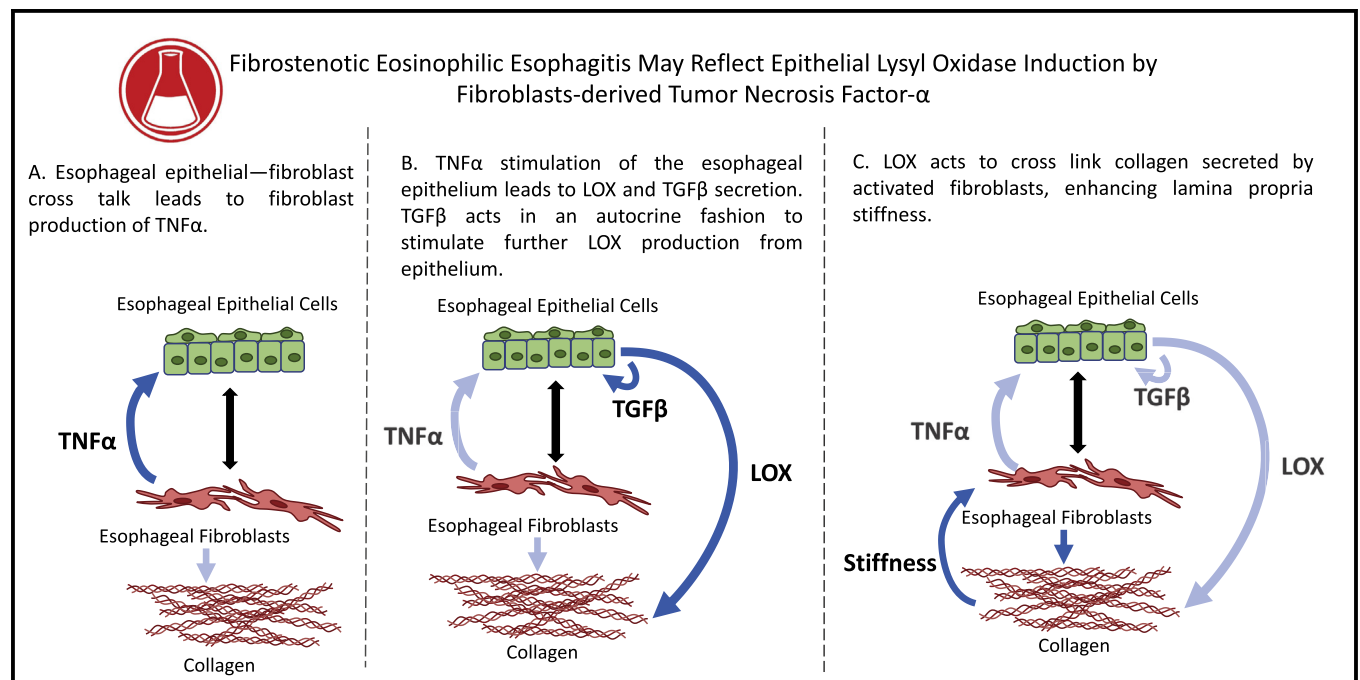


Fibrostenotic eosinophilic esophagitis might reflect epithelial lysyl oxidase induction by fibroblast-derived TNF- α

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GRAPHICAL ABSTRACT



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Background: Fibrosis and stricture are major comorbidities in patients with eosinophilic esophagitis (EoE). Lysyl oxidase (LOX), a collagen cross-linking enzyme, has not been investigated in the context of EoE.

Objective: We investigated regulation of epithelial LOX expression as a novel biomarker and functional effector of fibrostenotic disease conditions associated with EoE.

Methods: LOX expression was analyzed by using RNA-sequencing, PCR assays, and immunostaining in patients with EoE; cytokine-stimulated esophageal 3-dimensional organoids; and fibroblast–epithelial cell coculture, the latter coupled with fluorescence-activated cell sorting.

Results: Gene ontology and pathway analyses linked TNF- α and LOX expression in patients with EoE, which was validated in independent sets of patients with fibrostenotic conditions. TNF- α -mediated epithelial LOX upregulation was recapitulated in 3-dimensional organoids and coculture experiments. We find that fibroblast-derived TNF- α stimulates epithelial LOX expression through activation of nuclear factor κ B and TGF- β -mediated signaling. In patients receiver operating characteristic analyses suggested that LOX upregulation indicates disease complications and fibrostenotic conditions in patients with EoE.

Conclusions: There is a novel positive feedback mechanism in epithelial LOX induction through fibroblast-derived TNF- α secretion. Esophageal epithelial LOX might have a role in the development of fibrosis with substantial translational implications. (J Allergy Clin Immunol 2019;■■■:■■■-■■■.)

Key words: Eosinophilic esophagitis, lysyl oxidase, TNF- α , TGF- β , coculture, fibrosis

Eosinophilic esophagitis (EoE) is a food allergen-triggered, cytokine-mediated chronic inflammatory disease marked by mass migration of eosinophils and other immune cell types to the esophagus.^{1,2} Untreated inflammation in patients with EoE often causes tissue remodeling in the esophageal mucosa, leading to fibrosis and esophageal dysfunction evident clinically by dysphagia, food impactions, and strictures. The most serious consequence of esophageal remodeling and fibrostenosis is the extremely narrow-caliber esophagus, which represents 9% of patients with EoE and is associated with therapy resistance.³ Diagnosis of EoE is made based on upper endoscopy and biopsy results; however, histologic diagnosis of fibrosis is often suboptimal because of limited sampling of the subepithelial lamina propria compartment in biopsy specimens.⁴ Currently, there is no tissue biomarker available to predict fibrostenotic conditions associated with EoE.

TGF- β is the major cytokine that mediates EoE-related lamina propria remodeling along with T_H2 cytokines, including IL-4 and IL-13.⁵⁻⁷ These cytokines promote fibrosis by activating lamina propria fibroblasts to produce collagen and fibronectin, increasing eosinophil adherence to tissue and enhancing fibroblast contractility.^{6,8} Additionally, cross-talk between esophageal epithelial cells and fibroblasts leads to robust fibroblast production of TNF- α ,^{8,9} a profibrogenic cytokine that has been implicated in fibrotic disease conditions of the liver, intestine, and kidney¹⁰⁻¹²; however, the pathogenic role of TNF- α in patients with EoE remains elusive. In response to these cytokines, stromal fibroblasts are activated to deposit and construct networks of excess collagen and fibronectin in the lamina propria, leading to increased

Abbreviations used

3D: Three-dimensional
EoE: Eosinophilic esophagitis
FACS: Fluorescence-activated cell sorting
GFP: Green fluorescent protein
IHC: Immunohistochemistry
LOX: Lysyl oxidase

esophageal stiffness.¹³ In turn, stiffness itself contributes to fibroblast activation to express greater amounts of α -smooth muscle actin,¹⁴ a marker of fibroblast activation, and generate enhanced traction forces. Nevertheless, little is known about the molecular mechanisms regulating tissue stiffness in patients with EoE.

The process of collagen cross-linking involves extracellular modifications that result in increased stiffness. Lysyl oxidase (LOX) is a copper-dependent enzyme that acts on extracellular collagen to form intramolecular and intermolecular cross-links between neighboring collagen molecules, forming collagen fibers. LOX-induced collagen cross-linking contributes to the progression of cardiac and liver fibrosis^{15,16}; however, nothing is currently known about the role of LOX in esophageal fibrosis and how it might influence tissue stiffness in the context of EoE.

Here we analyzed well-annotated RNA-sequencing data of esophageal biopsy specimens from patients with EoE to identify unique activation of the TNF- α pathway and LOX in patients with EoE. We find that LOX can serve as a novel tissue biomarker for fibrostenotic disease in patients with EoE. Using an innovative coculture system of esophageal epithelial cells and fibroblasts, as well as the esophageal 3-dimensional (3D) organoid system with a recapitulation of human physiology and pathology, we examined the crosstalk between esophageal epithelial cells and fibroblasts to find that fibroblast-derived TNF- α perpetuates epithelial cell TGF- β signaling and LOX expression.

METHODS

Human subjects and endoscopic esophageal biopsy specimens

In accordance with institutional review board standards and guidelines at the Children's Hospital of Philadelphia and the Hospital of the University of Pennsylvania, after informed consent, esophageal biopsy specimens were taken from patients undergoing esophagogastroduodenoscopy as part of routine care. Subjects were classified per 2011 EoE clinical guidelines.¹⁷ Subjects who met clinical criteria and had histologic presence of 15 or more esophageal epithelial eosinophils per high-power field were given a diagnosis of active EoE. Patients with EoE who demonstrated resolution of histologic inflammation and eosinophilia documented on previous endoscopy were designated as EoE inactive (<15 eosinophils/high-power field). Subjects without EoE had no previous diagnosis of EoE and reported symptoms warranting esophagogastroduodenoscopy but demonstrated no histopathologic abnormalities. Subjects with proton pump inhibitor-responsive esophageal eosinophilia¹⁴ were excluded. Subjects with a history of inflammatory bowel disease, celiac disease, gastrointestinal bleeding, or any other acute or chronic intestinal disorders were excluded from recruitment. Patients' demographics are provided in [Tables I and II](#).

RNA-sequencing data analyses

Raw RNA-sequencing data on esophageal biopsy specimens with quality scores (GSE58640)¹⁸ representing 10 patients with active EoE and 6 healthy

TABLE I. Patients' demographics for the RT-PCR cohort

	Control cohort (n = 34)	Inactive cohort (n = 32)	Active cohort (n = 33)
Median age (y [range])	11.5 (1.5-67)	10.5 (3-50)	15 (5-48)
Male sex	21 (62%)	27 (84%)	22 (67%)
History of impaction(s)	1 (3%)	7 (22%)	15 (45%)
History of stricture(s)	0 (0%)	3 (9%)	2 (6%)
Mean eosinophils/high-power field (range)	0	2 (0-12)	48 (18-116)
Proton pump inhibitor	32 (94%)	29 (91%)	31 (94%)
Topical swallowed steroids	0 (0%)	8 (25%)	5 (15%)
Diet	0 (0%)	23 (72%)	19 (58%)

TABLE II. Patients' demographics for IHC cohorts

	Control cohort (n = 5)	Inactive cohort (n = 4)	Active cohort (n = 6)
Median age (y [range])	7 (4-10)	6 (2-10)	12 (10-15)
Male sex	4 (80%)	3 (75%)	5 (83%)
History of impaction(s)	1 (20%)	1 (25%)	1 (17%)
History of stricture(s)	0 (0%)	0 (0%)	0 (0%)
Mean eosinophils/high-power field (range)	0	5.5 (0-10)	28.5 (15-50)
Proton pump inhibitor	5 (100%)	4 (100%)	6 (100%)
Topical swallowed steroids	0 (0%)	0 (0%)	1 (17%)
Diet	0 (0%)	3 (75%)	0 (0%)

control subjects available at the National Center for Biotechnology Information Gene Expression Omnibus database were downloaded and aligned to the human genome GRCh38.p7 by using the STAR aligner (v252b).¹⁹ Genomically mapped reads were counted against reference genes as annotated in Genecode (version 25)²⁰ by using htseq-count.²¹ One sample from a patient with EoE (GSM1415921, EoE_803) was excluded from further analyses because of a low number of mapped reads. Genes were tested for differential expression between patients with EoE and control subjects by using DESeq2,²² yielding fold changes, *P* values, and false discovery rate-adjusted *P* values for each gene. Gene ontology analysis was performed with the online bioinformatics software DAVID 6.8.²³ The Benjamini-Hochberg method for multiple hypothesis testing was used. Functional annotation clustering was performed on protein-coding genes upregulated by more than 5-fold in the EoE sample set (false discovery rate, <0.1).²⁴⁻²⁶

Quantitative RT-PCR assays

RNA was isolated from esophageal biopsy specimens for quantitative real-time RT-PCR, as described previously,¹⁴ by using TaqMan Gene Expression Assays (Thermo Fisher Scientific, Waltham, Mass) for *LOX* (Hs00942483) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Hs99999905_m1), the latter as an internal control gene.

Cell culture and 3D esophageal organoids

The immortalized normal human esophageal epithelial cell line EPC2-hTERT and a derivative expressing green fluorescent protein (GFP; EPC2-hTERT-GFP) and primary normal fetal esophageal FEF3 fibroblasts and a derivative expressing tdTomato fluorescent protein (FEF3-tdTomato) were grown at 37°C in a humidified 5% CO₂ incubator, as described previously.^{27,28} Primary esophageal epithelial cultures from patients with EoE and subjects without EoE were used at passage 3, as described previously.²⁵ Cells were stimulated with TGF-β or TNF-α, as indicated in monolayer culture.

For media-swap experiments, conditioned epithelial medium was collected from confluent EPC2-hTERT cells grown in complete keratinocyte serum-free medium and used to stimulate confluent fibroblasts. Media were supplemented with 10% FBS before stimulation. After 24 hours, fibroblast-conditioned medium was used to stimulate fresh EPC2-hTERT cells for 24 hours alone and in the presence of infliximab (anti-TNF-α antibody, 1 μg/mL).

For coculture experiments, equal numbers (2.5×10^5) of EPC2-hTERT-GFP and FEF3-tdTomato cells were seeded simultaneously in 60-mm dishes and grown in keratinocyte serum-free medium supplemented with 1% FBS (Thermo Fisher Scientific) for 24 hours.

Human and murine esophageal 3D organoids were generated and subjected to morphologic analyses after TNF-α stimulation, as described previously.^{29,30} In brief, esophageal organoids were grown for 5 days before addition of 5 ng/mL TNF-α. Organoids were recovered by digesting with 1 U/mL Dispase I (BD Biosciences, San Jose, Calif) and fixed overnight in 4.0% paraformaldehyde. Specimens were embedded in 2.0% Bacto-Agar:2.5% gelatin before paraffin embedding or RNA was isolated for PCR.

Flow cytometry and fluorescence-activated cell sorting

Flow cytometry and fluorescence-activated cell sorting (FACS) were performed, as described previously.³¹ In brief, cells were trypsinized and resuspended in FACS buffer (PBS supplemented with 1% BSA). 4',6-Diamidino-2-phenylindole was used to determine cell viability. FACS-Calibur or LSRII (BD Biosciences) and FlowJo (TreeStar, Ashland, Ore) software were used for flow cytometry. The FACS Vantage SE and FACS Aria II (BD Biosciences) were used to purify GFP-positive EPC2-hTERT cells and tdTomato-positive FEF3 fibroblasts from the coculture of epithelial and fibroblast cells for RNA purification. Flow cytometry for GFP and tdTomato was repeated for each condition at least 3 times.

Transcription factor assay

By using the Signal Reporter Assay kit (Qiagen, Venlo, The Netherlands), EPC2-hTERT cells were transfected and stimulated with TNF-α for 24 hours, and the luciferase assay was performed, according to the manufacturer's instructions. Lysates were assessed for relative luciferase activity compared with unstimulated control values. Luciferase reporter assays for SMAD3 activity were carried out, as previously described.²³ Briefly, 2×10^5 EPC2-hTERT cells were seeded in 24-well plates. Two hundred nanograms of 3TP-lux³² and 5 ng of pRL-SV40 (Promega, Madison, Wis) were transfected overnight by using

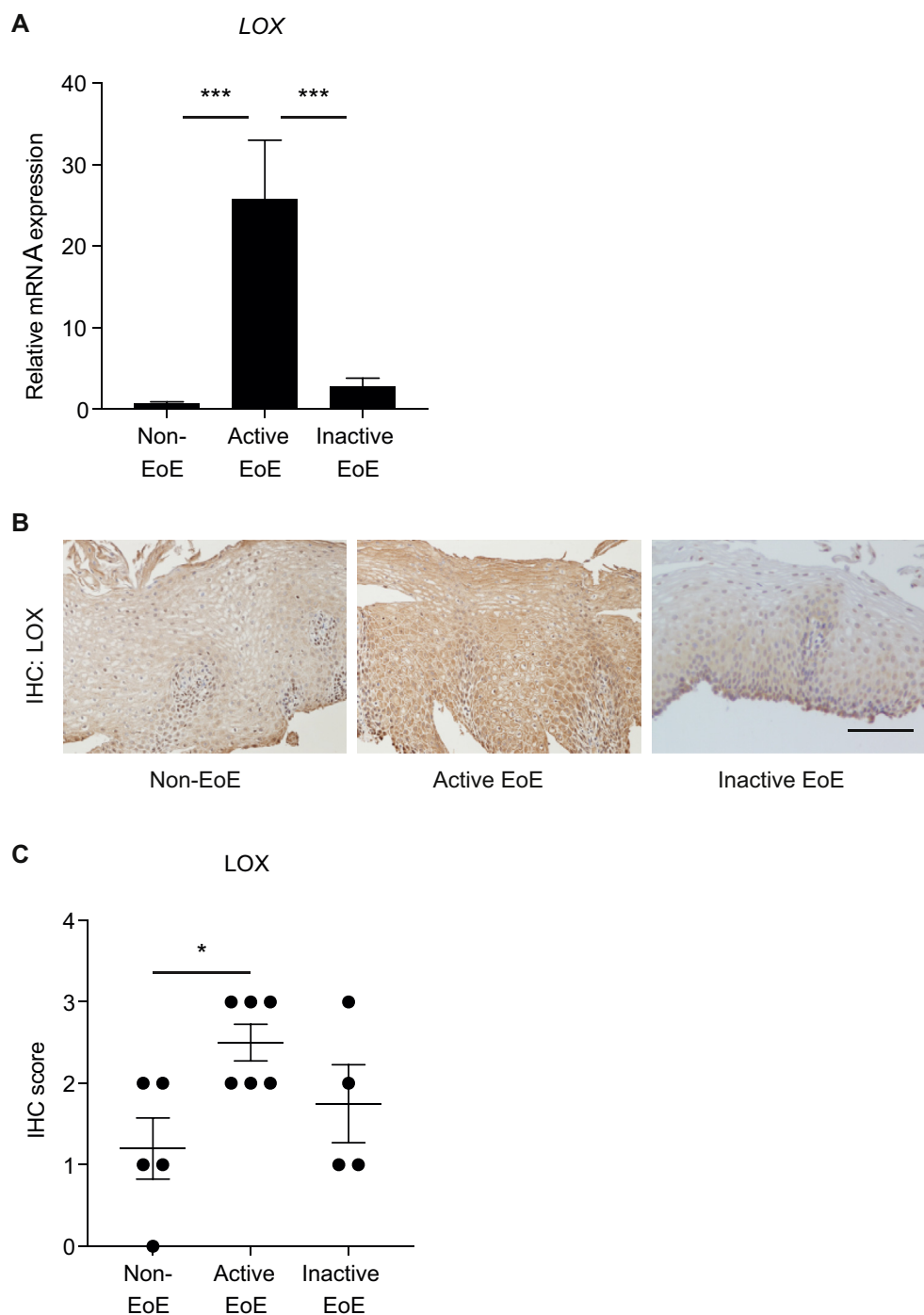


FIG 1. LOX expression is increased in patients with active EoE. Quantitative RT-PCR was performed to evaluate *LOX* mRNA expression from human esophageal biopsy specimens. **A**, *LOX* mRNA expression is increased in patients with active EoE (n = 33) compared with healthy subjects (n = 34) and patients with inactive EoE (n = 30). ANOVA was performed with multiple comparisons. **B**, IHC staining for LOX was performed, with representative images shown. Scale bar = 100 μ m. **C**, Histogram representing average LOX score (0-3) for healthy subjects (n = 5), patients with active EoE (n = 6), and patients with inactive EoE (n = 4). * P < .05 and *** P < .001.

Lipofectamine LTX Reagent with PLUS Reagent (Life Technologies, Grand Island, NY). The following day, cells were incubated with fresh media containing TNF- α for 48 hours. Luciferase activities were determined by using the Dual-Glo Luciferase Assay System (Promega), according to the manufacturer's recommendations, and the GloMax-Multi Detection

System (Promega). Cells were washed twice with 1 $\times$ PBS, lysed with 1 $\times$ Passive Lysis Buffer, and assayed in Microtiter White Microtiter Plates (Thermo). Firefly luciferase activities were normalized with Renilla luciferase activities before determining the means of technical triplicates. Variation between transfections did not exceed 15%.

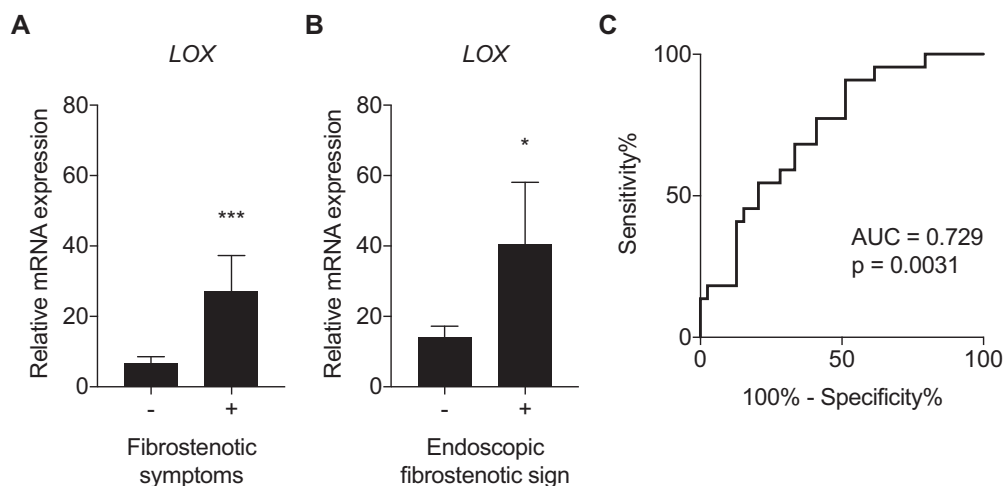


FIG 2. LOX expression increased in patients with fibrostenotic EoE. RNA from biopsy specimens from patients with EoE with and without disease complications (stricture or endoscopically removed food impaction) was isolated, and quantitative RT-PCR was performed. **A**, Those with complications ($n = 22$) were found to have enhanced LOX expression compared with those with uncomplicated disease ($n = 39$). **B**, LOX expression was significantly increased in patients with fibrostenotic endoscopic findings (rings and narrowing, $n = 12$) compared with that in patients with inflammatory endoscopy findings (furrows, exudates, and normal; $n = 31$). **C**, Receiver operator characteristics¹⁶ representing LOX expression and fibrostenotic complications. AUC, Area under the curve. * $P < .05$ and *** $P < .001$.

Immunohistochemistry

Paraffin-embedded biopsy specimens were serially sectioned and subjected to hematoxylin and eosin staining and immunohistochemistry (IHC), as described previously.^{23,27,29-31,33} In brief, sections were incubated with rabbit anti-LOX (1:100; rabbit polyclonal antibody NB100-2527; Novus Biological, Centennial, Colo)³⁴⁻³⁶ overnight at 4°C. Stained objects were captured and imaged with a Nikon Microphot microscope with a digital camera (Nikon, Shinagawa, Japan). IHC slides were evaluated by a pathologist (A.K.S.) blinded to molecular and clinical data to score for LOX intensity. A visual semiquantitative scoring from 0 to 3 was used for evaluation of IHC cytoplasmic staining intensity, where 0 is no staining, 1 is weak staining, 2 is moderate staining, and 3 is strong staining.

Immunoblotting

Cells in monolayer cultures were lysed and subjected to immunoblotting, as previously described.^{8,30} The rabbit mAb anti-Smad2/3 D7G7 (Cell Signaling Technology, Danvers, Mass), rabbit monoclonal anti-phospho-SMAD2/SMAD3 D27F4 (Cell Signaling Technology), rabbit polyclonal anti-LOX 102M4756V (Sigma-Aldrich, St Louis, Mo), and mouse monoclonal anti- β -actin AC-74 (1:10,000; Sigma-Aldrich) were used as primary antibodies. β -Actin served as a loading control.

Statistical analysis

Data are presented as means $\pm$ SEMs or means $\pm$ SDs and were analyzed by using the 2-tailed Student t test or ANOVA, where applicable. A P value of less than .05 was considered significant. Data were analyzed with the software package Prism (GraphPad Software, La Jolla, Calif). All authors had access to the study data and reviewed and approved the final manuscript.

RESULTS

RNA-sequencing data reveal LOX upregulation and unique TNF- α pathway enrichment in biopsy specimens from patients with EoE

Gene expression profiling of EoE has been extensively carried out in biopsy specimens from patients with EoE. Sherrill

et al¹⁸ carried out RNA-sequencing studies to identify novel EoE-associated transcriptomes, complementing previous microarray studies that identified EoE-enriched genes, including eotaxin 3, *POSTN*, and *TNFAIP6*.³⁷ Although earlier studies focused on potential immunomodulators, immune cell-specific genes and noncoding RNAs associated with EoE,¹⁸ or those related to genetic variants,³⁸ we mined the well-annotated RNA-sequencing data (GSE58640) for genes relevant to fibrosis and tissue remodeling. Gene ontology analysis revealed that the top 2 enriched clusters contain biological processes involving collagen catabolism and extracellular matrix disassembly (see Table E1 in this article's Online Repository at www.jacionline.org). LOX was present in these 2 clusters, prompting us to focus specifically on its role in patients with EoE (see Table E2 in this article's Online Repository at www.jacionline.org for the full list of genes in the top cluster).

RNA-sequencing analysis revealed that LOX expression was significantly upregulated in the cohort of patients with EoE compared with control subjects. We found that LOX expression was 11.3-fold increased in patients with active EoE ($P = 1.19 \times 10^{-8}$). Ingenuity pathway analysis revealed that genes in the TNF- α pathway were among the most highly enriched in biopsy specimens from patients with EoE ($P = 1 \times 10^{-40}$). There were 328 TNF- α target genes enriched in biopsy specimens from patients with active EoE, suggesting robust TNF- α activation in the population with EoE (see Table E3 in this article's Online Repository at www.jacionline.org), a novel premise. Based on these findings, we sought to further investigate the role of LOX and TNF- α in the pathogenesis of EoE-related fibrosis.

LOX is upregulated in the esophagus in patients with EoE

To validate sequencing results in an independent patient cohort, we performed real-time RT-PCR analyses on esophageal biopsy

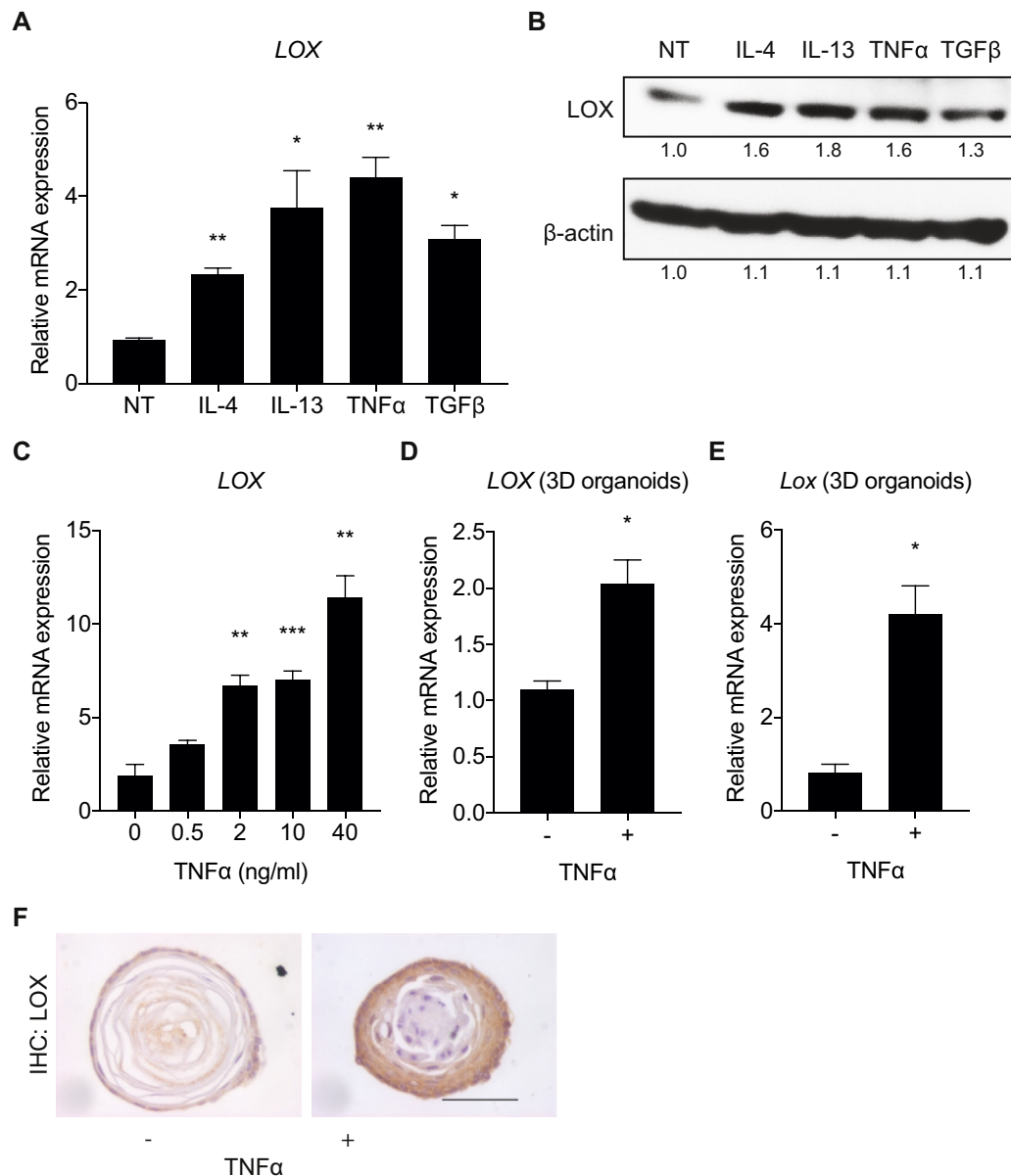


FIG 3. EoE milieu and TNF- α upregulate LOX expression in esophageal epithelial cells. **A** and **B**, EPC2-hTERT cells were treated with the indicated cytokines for 24 hours and evaluated by using PCR (Fig 3, **A**) and Western blotting (Fig 3, **B**). Densitometry was performed on Western blots. Numbers under each band represent the signal intensity relative to the no treatment (NT) set as 1. **C**, EPC2-hTERT cells were treated with increasing doses of TNF- α . **D** and **E**, Human and murine esophageal epithelial cells were isolated and grown in matrigel to support organoid formation. Organoids were stimulated with TNF- α , and quantitative RT-PCR was performed for LOX in human (Fig 3, **D**) and murine (Fig 3, **E**) organoids, respectively. **F**, IHC for LOX was performed on murine organoids stimulated with TNF- α . Scale bar = 50 μ m. Data represent 2 or more biologic replicates with similar results. * $P < .05$, ** $P = .01$, and *** $P < .001$.

specimens collected from patients with active and inactive EoE, as well as control subjects without EoE (Fig 1, **A**). We found that patients with active EoE had significantly enhanced LOX mRNA expression compared with control subjects and patients with inactive EoE, indicating that the unique inflammatory milieu in patients with active disease is driving LOX production in the esophagus. Interestingly, those with inactive disease also had significantly increased LOX expression compared with control subjects, suggesting that LOX upregulation is mainly attributed to epithelial cells rather than nonepithelial cells, such as

infiltrating immune cells. This was corroborated by using IHC (Fig 1, **B**), which revealed that patients with active EoE exhibited significantly increased LOX immunoreactive protein expression compared with that in subjects without EoE. LOX expression is strongest in the epithelium of the esophagus.

Fibrostenotic EoE shows upregulation of LOX

Recently, we have shown that in patients with pediatric EoE, there is decreased esophageal distensibility compared with that in

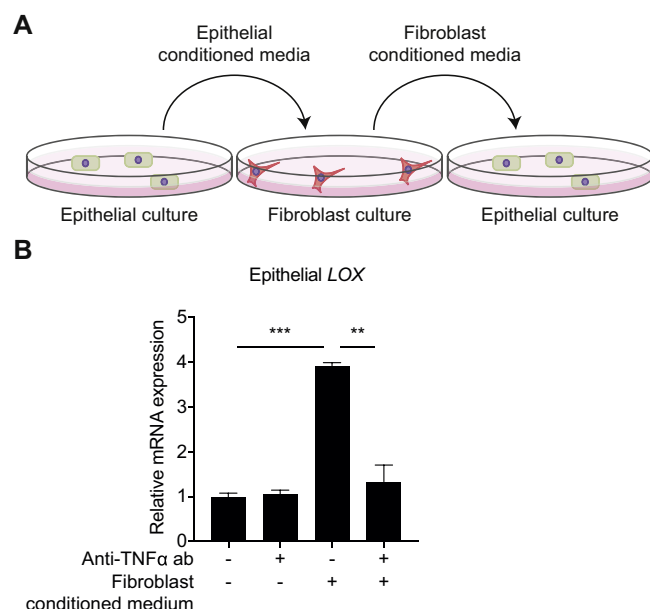


FIG 4. Conditioned media enhances LOX expression *in vitro*. **A**, Schematic of experimental design. Media from esophageal epithelial cells was placed on esophageal fibroblasts. After 24 hours, fibroblast-conditioned media was placed on fresh epithelial cells in the presence of TNF- α inhibitory antibody. **B**, After 24 hours of stimulation, quantitative RT-PCR was performed for LOX. Data represent 3 biologic replicates with similar results. ** $P = .01$ and *** $P < .001$.

subjects without EoE⁴ by using functional endoluminal imaging. However, there is no standard procedure to monitor or detect esophageal fibrosis. Therefore we sought to determine how LOX expression varied depending on the presence of fibrostenotic disease. Patients with EoE were grouped based on presentation of disease complications or endoscopic evaluation. Fibrostenotic complications of EoE were defined as patients with a history of stricture or food impaction requiring endoscopic bolus removal. Endoscopic fibrostenosis was defined as the presence of stricture or rings. We then analyzed LOX expression in these groups and found a significant upregulation in patients with fibrostenotic disease (both clinically and endoscopically) when compared with those with nonfibrostenotic EoE (Fig 2, A and B).

We then determined whether LOX mRNA expression levels in endoscopic biopsy specimens could be used as a molecular marker of fibrostenotic disease. Using a relative fold increase in LOX mRNA expression of 5.233 compared with a healthy control group without EoE as a cutoff point to generate a receiver operating characteristic curve (Fig 2, C),¹⁶ patients were found to be classified as having fibrostenotic EoE with 52.17% sensitivity, 79.49% specificity, and positive and negative predictive values of 68% and 76%, respectively, indicating that LOX upregulation in predominantly epithelial endoscopic biopsy specimens predicts fibrostenotic disease activity. Specifically, LOX upregulation in biopsy specimens can hold unprecedented implications in EoE diagnosis and therapies.

EoE-relevant cytokines induce LOX

To determine which factors contribute to increased LOX expression in patients with EoE, we stimulated EPC2-hTERT cells with the EoE-relevant cytokines IL-4, IL-13, TNF- α , and

TGF- β . We found that each of these cytokines significantly induced LOX RNA expression *in vitro*, as well as LOX protein expression, using Western blotting (Fig 3, A and B). TNF- α produced the most robust response and showed synergistic effects with each of the other EoE-relevant cytokines (see Fig E1 in this article's Online Repository at www.jacionline.org) when costimulated. Dose-dependent induction of LOX was observed further in the context of TNF- α stimulation (Fig 3, C). Using an *ex vivo* 3D model of EoE that recapitulates epithelial changes compatible with basal zone hyperplasia on treatment with EoE-relevant cytokines,²⁹ we found enhanced LOX RNA expression in response to TNF- α (Fig 3, D and E) in both murine and human-derived esophageal organoids, and this was confirmed by using IHC in murine organoids (Fig 3, F). This result also confirmed epithelial cell induction of LOX because 3D organoids were grown in the absence of nonepithelial cell components.

Epithelial-stromal fibroblast crosstalk induces epithelial LOX

We have previously shown that TNF- α is produced by esophageal fibroblasts stimulated with conditioned media from esophageal epithelial cell cultures³³ and that TNF- α in turn induces epithelial cells to undergo functional changes, such as increased collagen production and contraction.⁸ Crosstalk between the epithelial cells and fibroblasts induces a profibrotic milieu, even in the absence of inflammatory cells. Therefore we hypothesized that crosstalk between these cell types can induce LOX *in vitro*.

We stimulated EPC2-hTERT cells with media from esophageal fibroblasts (FEFs) that had been primed with conditioned epithelial media (Fig 4, A) and found that the epithelial cells stimulated with the fibroblast-conditioned media displayed increased LOX expression (Fig 4, B). To explore the role of TNF- α in LOX production, we then treated the EPC2-hTERT cells with the anti-TNF- α antibody infliximab. We found that treatment with anti-TNF- α caused a significant decrease in LOX expression, suggesting that fibroblast-derived TNF- α stimulates epithelial cell LOX expression.

Coculture of fibroblasts and epithelial cells induces LOX in a TNF- α -dependent manner

We examined epithelial cell expression of LOX in the presence of esophageal fibroblasts through direct coculture experiments. To unequivocally determine cell type-specific gene expression in cocultured epithelial cells and fibroblasts, we used the normal esophageal epithelial cell line EPC2-hTERT and FEF3 fibroblasts permanently labeled with GFP and tdTomato, respectively. After culturing together for 24 hours, cells were subjected to separation of tdTomato-positive FEF3 and GFP-positive EPC2-hTERT cells through FACS. We performed purity analysis to confirm that populations of cells represent greater than 99% of the intended cell fraction (Fig 5, A). These purified cell populations were analyzed for LOX expression by means of real-time RT-PCR assays. LOX expression in EPC2-hTERT cells was robustly augmented in the presence of fibroblasts (Fig 4, B). This result was confirmed with 2 independent biopsy-derived primary esophageal epithelial cell cultures (EPC-A and EPC-B; Fig 5, B).²⁵ We then used anti-TNF- α antibody in the coculture system and found decreased epithelial cell LOX production, suggesting TNF- α -mediated

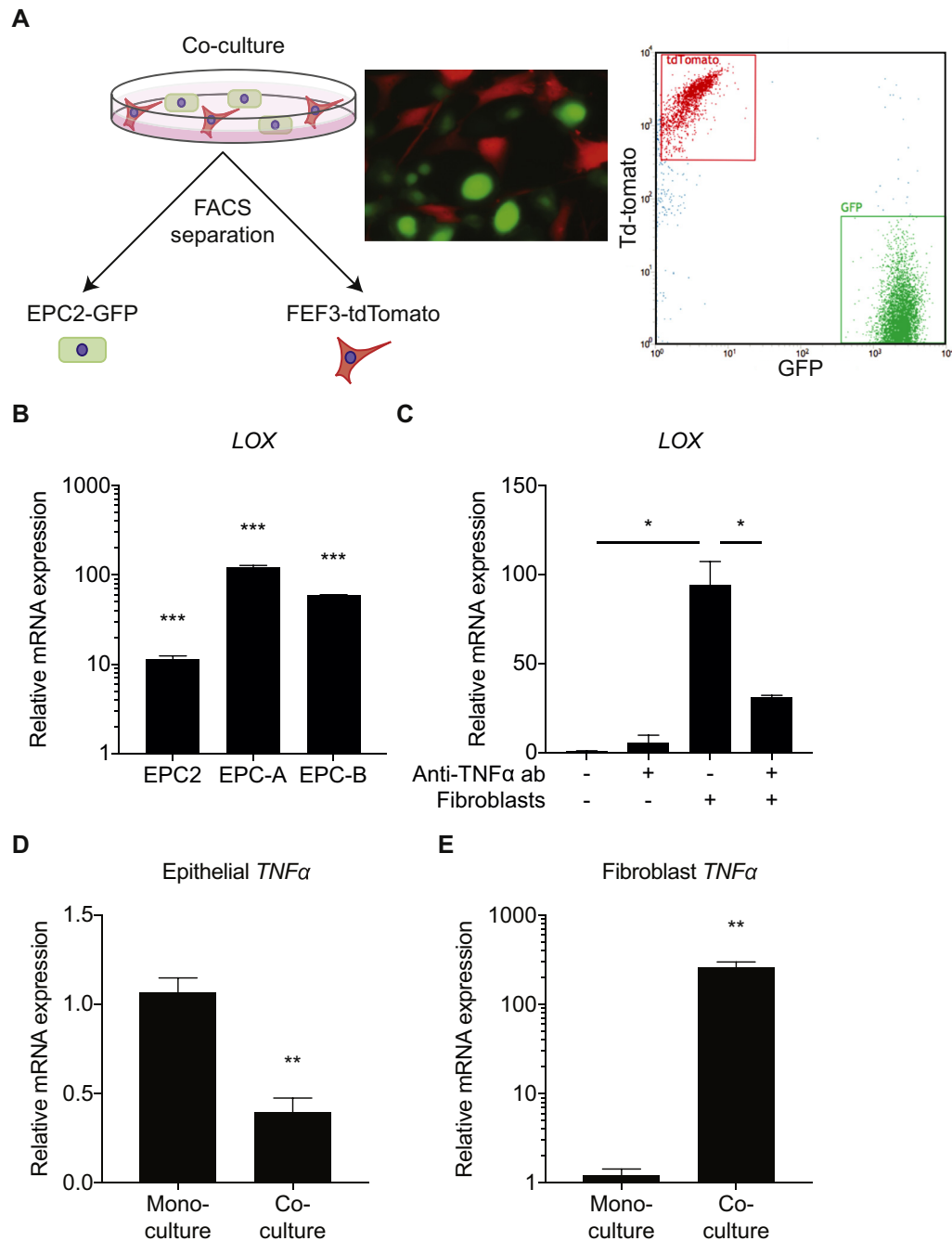


FIG 5. Coculture of esophageal fibroblasts and epithelial cells induces LOX expression. **A**, FEF-tdTomato and EPC2-GFP cells were grown together for 24 hours and separated by using FACS. **B**, Quantitative RT-PCR (qRT-PCR) for *LOX* was performed on EPC2-hTERT cells after coculture and shown relative to monoculture (coculture/monoculture). This was validated with 2 independent primary esophageal epithelial cell cultures derived from patients' biopsy specimens: EPC-A and EPC-B. **C**, Anti-TNF- α antibody was added to coculture, and qRT-PCR was performed for *LOX*. **D**, qRT-PCR for TNF- α was performed on EPC2-hTERT cells (Fig 5, *D*) and FEF3 fibroblasts (Fig 5, *E*) cells after coculture. Data represent 3 biologic replicates with similar results. * $P < .05$, ** $P = .01$, and *** $P < .001$.

regulation of *LOX* expression in esophageal epithelial cells (Fig 5, *C*). In addition, we analyzed the FEF3 compartment for *LOX* production. Although the fibroblasts expressed *LOX*, it was not significantly influenced by epithelial cells (see Fig E2 in this article's Online Repository at www.jacionline.org).

To confirm that TNF- α was fibroblast derived, we performed RT-PCR on epithelial and fibroblast compartments after coculture. We found that epithelial TNF- α was significantly decreased after 24 hours of coculture with fibroblasts (Fig 5, *D*). Conversely, fibroblasts cocultured with epithelial cells show a robust

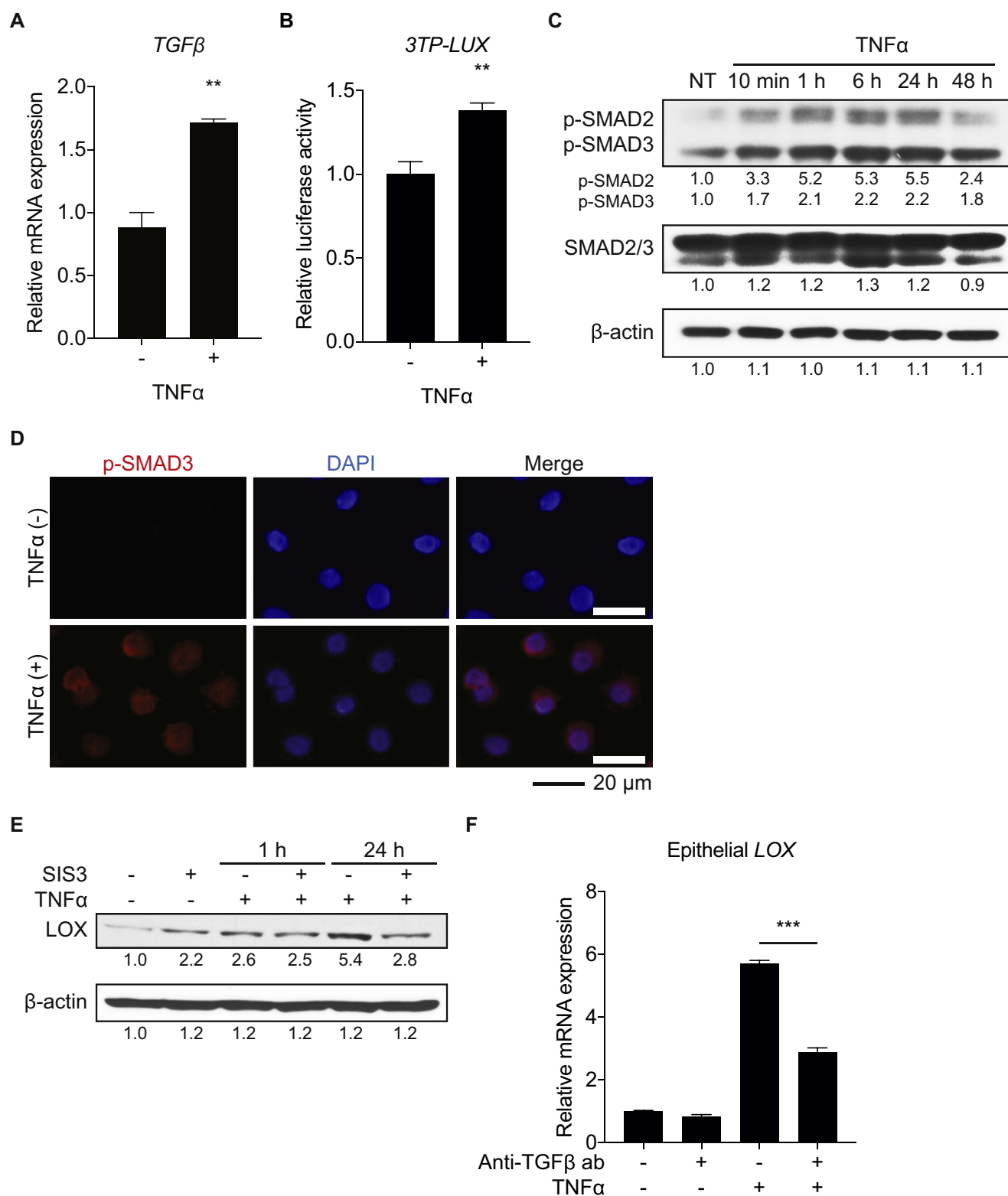


FIG 6. TNF- α stimulation of TGF- β contributes to LOX expression. **A-D**, TNF- α stimulation of the esophageal epithelial monolayer induces TGF- β expression (Fig 6, A); SMAD activity, as measured by using the luciferase assay (Fig 6, B); increased SMAD phosphorylation (Fig 6, C); and nuclear localization of phosphorylated SMAD3 (Fig 6, D). DAPI, 4',6-Diamidino-2-phenylindole. **E**, Inhibition of SMAD with SIS3 inhibited LOX production in TNF- α -stimulated EPC2-hTERT cells by means of immunoblotting. **F**, Addition of anti-TGF- β antibody (1D11) to TNF- α -stimulated EPC2-hTERT cells abrogates LOX expression. Isotype antibody used as control did not stimulate LOX expression (data not shown). Numbers under each band represent the signal intensity relative to the no treatment (NT) set as 1. Data represent 2 or more biologic replicates with similar results. ** P = .01 and *** P < .001.

induction of TNF- α , confirming our previous results (Fig 5, E).⁹ This suggests that fibroblast-derived TNF- α induces epithelial LOX production in our model system.

TNF- α stimulation of TGF- β contributes to LOX expression

TNF- α and TGF- β have a controversial relationship. TNF- α has been shown to both enhance and inhibit TGF- β in an organ-specific context.^{39,40} Because the relationship between TNF- α in TGF- β induction in the esophagus remains elusive and canonical TGF- β signaling^{41,42} is known to stimulate LOX production,⁴³⁻⁴⁵ we hypothesized that the TNF- α -rich EoE microenvironment can induce epithelial production of TGF- β ^{46,47} to further perpetuate LOX production. We stimulated EPC2-hTERT cells with TNF- α and found increased expression of TGF- β (Fig 6, A).

Using reporter transfection assays with a panel representing multiple transcription factor responsive elements, TNF- α -stimulated EPC2-hTERT cells revealed SMAD-mediated transcriptional activation (data not shown). To confirm these findings, we performed luciferase assays, finding that TNF- α stimulation significantly increased SMAD activity, SMAD phosphorylation, and nuclear translocation of phospho-SMAD (Fig 6, B-D). In addition, we used SIS3, a SMAD inhibitor, and costimulated with TNF- α . We found that LOX production was decreased by means of immunoblotting. Costimulation of EPC2-hTERT cells with TNF- α and anti-TGF- β antibody (1D11) significantly reduced LOX expression (Fig 6, D). These results suggest that epithelial LOX production is subjected to transcriptional regulation activated through a functional interplay between TNF- α and canonical TGF- β signaling.

DISCUSSION

Our data indicate that the level of LOX expression in esophageal tissue corresponds with fibrotic disease in patients with EoE. We have shown that there is increased epithelial LOX expression in patients with EoE compared with that seen in subjects without EoE. Most strikingly, LOX upregulation in endoscopic biopsy specimens from patients with EoE might implicate subepithelial tissue remodeling in the early stage of EoE progression and serve clinically as a biomarker of the fibrostenotic disease condition. We further defined the role of fibroblast-epithelial cell interaction in driving the production of LOX *in vitro* through activation of the TNF- α and TGF- β pathways.

The population with EoE encompasses a diverse constellation of symptoms, endoscopic findings, and histology that vary greatly based on age at diagnosis. Disease chronicity is thought to play a role in the development of fibrostenotic features of EoE.^{48,49} Defining fibrostenotic EoE before the onset of a true stricture or food impaction can be problematic in patients with EoE.⁴ The lamina propria is not interpretable by pathologists in up to 50% of biopsy specimens from pediatric patients with EoE because the deep subepithelial compartment is not procured when using relatively small endoscopy forceps.

This is the first study to identify a potential biomarker of fibrostenotic disease in patients with EoE, whereas several studies have proposed markers of disease activity.¹⁸ The relatively small sample size of our population might explain the lack of sensitivity of LOX; however, these data suggest that larger-scale

investigations are warranted. It might hold potential to detect prestenosis when used in conjunction with other genes or in conjunction with new functional esophageal tests, such as the EndoFLIP.^{4,50} Recent work by Rawson et al⁵¹ evaluated epithelial expression of plasminogen activator inhibitor 1 and found that it contributes to the fibrotic network in patients with EoE, correlating expression with vimentin, collagen 1, fibronectin, and matrix metalloproteases. Recently, we have shown that patients with fibrostenotic features of EoE, such as a history of stricture, impaction, or rings/narrowing on endoscopy, display decreased esophageal distensibility measured by using the EndoFLIP.⁴ Future longitudinal studies combining these transcriptional targets with functional esophageal testing will provide a better understanding of global disease activity and future risk of fibrostenosis, allowing for diagnosis before the onset of clinical complications. Additionally, we will consider disease chronicity, evaluating LOX over time and determining its variability in the treatment-resistant and treatment-responsive cohorts.

To our knowledge, this is the first time epithelial LOX expression has been associated with esophageal fibrosis and EoE. The most important findings of this study include a novel paradigm showing that disease-associated epithelial changes can influence the subepithelial fibrostenotic disease process through paracrine-mediated interplay between epithelial cells and fibroblasts under the EoE inflammatory milieu. Fibrosis is defined as inappropriate deposition of excessive amounts of collagen and fibrous tissue. Indeed, our gene ontology analyses of well-annotated RNA-sequencing data reveal molecular pathways essential in wound healing, in which genes encoding metalloproteases and periostin can play critical roles in addition to LOX (see Table E2). Our recent work has determined that the functional properties of collagen, specifically its resistance to degradation and material stiffness, is just as important as the quantity of collagen.¹⁴ Inhibiting LOX in murine models of atherosclerosis leads to decreased arterial stiffness despite high-fat diets and increased cholesterol levels.⁵² LOX-induced stiffening might even be the first step in fibroblast activation. Before the onset of fibrosis in rat models of liver disease, LOX-mediated tissue stiffening occurs and is then followed by fibroblast activation.⁵³ This observation is consistent with our previous reports that esophageal myofibroblast activation requires matrix stiffness.¹⁴ Taken together, LOX expression in the esophageal epithelium could propagate fibrosis, stimulating a positive feedback loop resulting in ongoing tissue stiffness and continued remodeling.

By mining RNA-sequencing data, for the first time, we have also defined in an unbiased approach the potential role of epithelial TNF- α signaling in EoE pathogenesis. Persad et al⁵⁴ previously described enhanced TNF- α -induced angiogenesis in patients with EoE. We have also previously implicated TNF- α in EoE-related esophageal fibrosis,^{8,9} where fibroblast-epithelial cell interactions appeared to drive epithelial-mesenchymal transition *in vitro* and TNF- α stimulation, resulting in enhanced epithelial contraction, migration, and collagen deposition. In this study we highlight another potential consequence of TNF- α enrichment: enhanced TGF- β signaling. TGF- β is often cited as the “master regulator”⁵⁵ of fibrosis. Through various activities, including activation of fibroblasts, stimulation of production of extracellular matrix, and stimulation of smooth muscle contraction,⁵⁶ TGF- β is likely responsible for many of the clinical symptoms of EoE. Here we show for the first time that the

esophagus in patients with EoE has a unique enrichment of TNF- α that contributes to fibrosis through canonical TGF- β stimulation.

TNF- α is known to contribute to fibrosis in other disease models.¹⁰ In the intestine there is clinical evidence that infliximab (chimeric anti-TNF- α mAb) not only decreases inflammation but also treats fibrostenotic Crohn disease.⁵⁷ Infliximab was studied in 3 patients with steroid-dependent EoE. After 2 doses of infliximab, tissue eosinophilia was not improved; however, two thirds of subjects had decreased symptoms of dysphagia.⁵⁸ Although the pilot study was hindered by its small study size and short duration of therapy, it brings to light the potential use of anti-TNF- α therapy for EoE-associated fibrosis.

Previously, we demonstrated that epithelial cell-fibroblast crosstalk through conditioned media induces secretion of the profibrotic cytokines IL-1 β and TNF- α . Transfer of fibroblast-conditioned medium onto epithelial cells increased expression of mesenchymal markers vimentin and α -smooth muscle actin and loss of E-cadherin in a TNF- α -dependent manner.⁹ Furthermore, when epithelial cells were stimulated with TNF- α , they underwent functional changes, including increased migration, contraction, and enhanced collagen expression.⁸ The results of our current study suggest that coculture (both directly and indirectly) can enhance tissue stiffness through LOX production. Future functional studies will seek to perform both gain of function and knockdown of LOX in the epithelial cells and evaluate the effects of coculture on fibroblast function, specifically spread, contraction, collagen production, and tissue stiffness.

Therapeutic interventions in patients with EoE are largely targeted at decreasing eosinophilic inflammation. However, the 2 major therapies, a 4-food elimination diet and topical viscous steroids, have remission rates ranging from 20% to 50%.⁵⁹ Because there is no US Food and Drug Administration-approved medication for EoE and response rates with current strategies are abysmal, targeting stiffness through LOX or TNF- α inhibition can have a role in preserving the swallowing function of those with treatment resistance and fibrostenosis.

Clinical implications: LOX expression is increased in patients with active EoE. Its expression in the esophageal epithelium is mediated by fibroblast-derived TNF- α and downstream TGF- β production.

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Supplementary Figure 1: EPC2-hTERT cells were co-stimulated with TNF α and either IL-13, IL-4 or TGF β . qRT-PCR was performed on epithelial cells after 24 hours and shown relative to unstimulated monolayer culture. ***p<0.001

Supplementary Figure 2: FEF-tdTomato and EPC2-GFP were grown together for 24 hours and separated by FACS. qRT-PCR for LOX was performed on FEF after co-culture and shown relative to monoculture. NS, not significant.

Figure S1
A

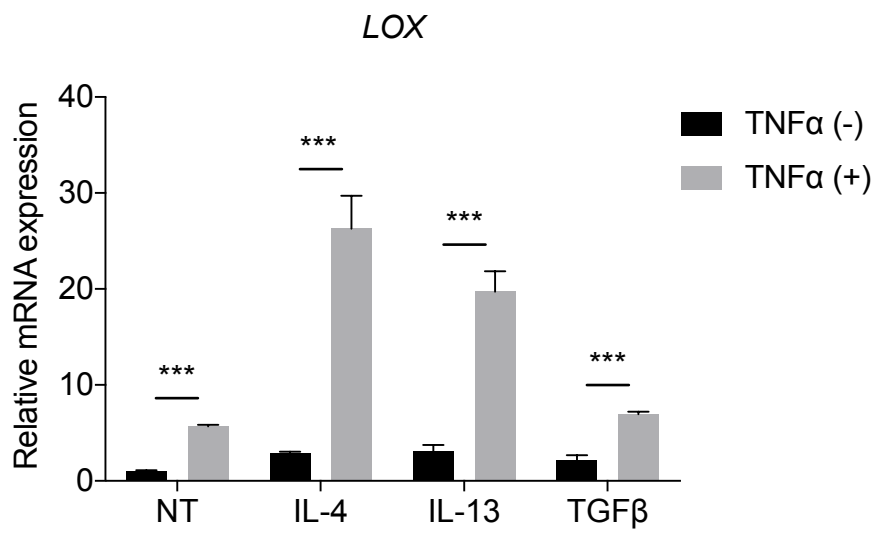


Figure S2
A

