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Increased Production of LIGHT by T Cells in Eosinophilic Esophagitis Promotes Differentiation of Esophageal Fibroblasts Toward an Inflammatory Phenotype

Mario C. Manresa[1,2,3], **Austin W. T. Chiang**[1,4], **Richard C. Kurten**[5], **Ranjan Dohil**[6], **Howard Brickner**[8], **Lucas Dohil**[1], **Rana Herro**[9], **Praveen Akuthota**[7,10], **Nathan E. Lewis**[1,4,11], **Michael Croft**[3,7,§], **Seema S. Aceves**[1,2,6,7,§]

[1]Department of Pediatrics, University of California, San Diego, San Diego

[2]Division of Allergy Immunology

[3]La Jolla Institute for Immunology, La Jolla, California

[4]Novo Nordisk Foundation Center for Biosustainability at the University of California, San Diego, San Diego, California

[5]Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Arkansas Children's Hospital Research Institute, Little Rock, Arkansas

[6]Rady Children's Hospital, San Diego

[7]Division of Gastroenterology, Department of Pediatrics, University of California, San Diego

[8]Department of Medicine, University of California, San Diego, San Diego, California

[9]Cincinnati Children's Hospital Medical Center, Immunobiology Division, Cincinnati, Ohio

Address correspondence to: Professor Seema S. Aceves, MD, PhD, University of California San Diego, Department of Pediatrics, 9500 Gilman Drive, La Jolla, San Diego, California. saceves@health.ucsd.edu.

[§]Authors share co-senior authorship.

CRediT Authorship Contributions

Mario C. Manresa, PhD (Conceptualization: Equal; Data curation: Lead; Formal analysis: Lead; Investigation: Lead; Methodology: Lead; Project administration: Lead; Supervision: Equal; Validation: Equal; Writing – original draft: Lead); Austin W.T. Chiang, PhD (Data curation: Equal; Formal analysis: Equal; Methodology: Equal; Software: Lead; Writing – original draft: Supporting); Richard C. Kurten, PhD (Methodology: Equal; Resources: Equal; Validation: Equal); Ranjan Dohil, MD (Methodology: Equal; Resources: Equal; Validation: Equal); Howard Brickner, MSc (Methodology: Equal; Resources: Equal; Validation: Equal); Lucas Dohil, BSc (Methodology: Equal); Rana Herro, PhD (Funding acquisition: Supporting); Praveen Akuthota, MD (Conceptualization: Supporting; Methodology: Supporting; Supervision: Equal); Nathan E. Lewis, PhD (Conceptualization: Supporting; Methodology: Supporting; Supervision: Equal); Michael Croft, PhD (Conceptualization: Lead; Formal analysis: Lead; Funding acquisition: Lead; Investigation: Supporting; Methodology: Supporting; Project administration: Lead; Resources: Supporting; Supervision: Lead; Validation: Lead; Writing – review & editing: Lead); Seema S. Aceves, MD, PhD (Conceptualization: Lead; Formal analysis: Lead; Funding acquisition: Lead; Investigation: Supporting; Methodology: Supporting; Project administration: Lead; Resources: Supporting; Supervision: Lead; Validation: Lead; Writing – review & editing: Lead).

Conflicts of interest

Ranjan Dohil and Seema S. Aceves and are co-inventors of oral viscous budesonide for eosinophilic esophagitis patented by the University of California, San Diego and licensed by Shire-Takeda. Michael Croft has patents on TNFSF14/LIGHT. Seema S. Aceves is a consultant for Regeneron, AImmune, Astellas, AstraZeneca, DBV, and Gossamer Bio. The remaining authors disclose no conflicts.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2020.07.035.

[10]Division of Pulmonary, Critical Care, and Sleep Medicine, University of California San Diego, La Jolla, California

[11]Department of Bioengineering, University of California, San Diego, San Diego, California

Abstract

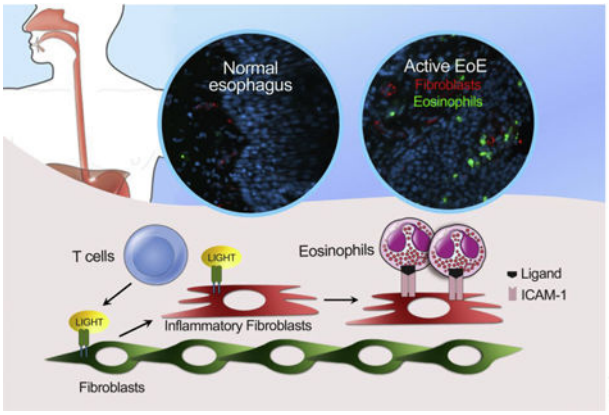
BACKGROUND & AIMS: Eosinophilic esophagitis (EoE) is an antigen-mediated eosinophilic disease of the esophagus that involves fibroblast activation and progression to fibrostenosis. Cytokines produced by T-helper type 2 cells and transforming growth factor beta 1 (TGFβ1) contribute to the development of EoE, but other cytokines involved in pathogenesis are unknown. We investigate the effects of tumor necrosis factor superfamily member 14 (TNFSF14, also called LIGHT) on fibroblasts in EoE.

METHODS: We analyzed publicly available esophageal $CD3^+$ T-cell single-cell sequencing data for expression of LIGHT. Esophageal tissues were obtained from pediatric patients with esophageal biopsy samples of healthy donors or patients with active EoE. Fibroblasts were cultured; incubated with TGFβ1 and/or LIGHT; and analyzed by RNA sequencing, flow cytometry, immunoblots, immunofluorescence, or reverse transcription polymerase chain reaction. Eosinophils were purified from peripheral blood of healthy donors, incubated with interleukin 5, cocultured with fibroblasts, and analyzed by immunohistochemistry.

RESULTS: LIGHT was up-regulated in the esophageal tissues from patients with EoE, compared with control individuals, and expressed by several T-cell populations, including T-helper type 2 cells. TNF receptor superfamily member 14 (TNFRSF14, also called HVEM) and lymphotoxin beta receptor are receptors for LIGHT that were expressed by fibroblasts from healthy donors or patients with active EoE. Stimulation of esophageal fibroblasts with LIGHT induced inflammatory gene transcription, whereas stimulation with TGFβ1 induced transcription of genes associated with a myofibroblast phenotype. Stimulation of fibroblasts with TGFβ1 increased expression of HVEM; subsequent stimulation with LIGHT resulted in their differentiation into cells that express markers of myofibroblasts and inflammatory chemokines and cytokines. Eosinophils tethered to esophageal fibroblasts after LIGHT stimulation via intercellular adhesion molecule-1.

CONCLUSIONS: T cells in esophageal tissues from patients with EoE express increased levels of LIGHT compared with control individuals, which induces differentiation of fibroblasts into cells with inflammatory characteristics. TGFβ1 increases fibroblast expression of HVEM, a receptor for LIGHT. LIGHT mediates interactions between esophageal fibroblasts and eosinophils via ICAM1. This pathway might be targeted for the treatment of EoE.

Graphical Abstrcat



Keywords

Fibrosis; Fibrogenesis; Immune Regulation; ICAM1; Eosinophilia

Eosinophilic esophagitis (EoE) is an allergen-triggered T-helper type 2 disease associated with robust tissue eosinophilia. EoE is increasing in prevalence, representing a $1.36 billion/year growing health care burden.[1,2] Unbridled inflammation leading to overactive wound healing causes fibrosis, a process common to a number of chronic inflammatory diseases, including asthma and inflammatory bowel disease.[3–5] In EoE, fibrosis leads to tissue remodeling with resultant esophageal rigidity and luminal narrowing, causing clinical dysphagia and food impactions.[2,3,6] Current EoE therapies have variable and limited effects on treating fibrostenotic EoE.[7] Tissue fibroblasts are likely central to fibrosis in EoE, shaping the extracellular matrix, but varying functions and phenotypes of fibroblasts in alternate disease states are just beginning to be appreciated. Although the profibrotic role of myofibroblasts associated with extracellular matrix production is expected in EoE, the potential function of fibroblasts as innate inflammation-organizing cells is unknown. Current data support the production of tumor necrosis factor (TNF) by esophageal fibroblasts, supporting their role as potential proinflammatory cells.[8] In other gastrointestinal diseases such as ulcerative colitis, single-cell analyses have shown the presence of fibroblasts with inflammatory properties.[9] Recent studies have also identified novel fibroblast phenotypes in rheumatoid arthritis as well as in skin wounds,[10,11] showing their ability to produce a wide range of mediators, including chemokines, adhesion molecules, and cytokines. This suggests their potential role in chemotaxis and activation of immune cells. However, the complex cytokine milieu that drives fibroblast differentiation is unclear.

Transforming growth factor-beta 1 (TGFβ1)–expressing cells are increased in the active EoE esophagus.[12,13] TGFbβ1 induces profibrotic gene expression in esophageal fibroblasts and, together with TNF, promotes collagen crosslinking.[8,14] In addition, TGFβ1 promotes a smooth muscle cell-like myofibroblast with contractile properties.[15,16] Moreover, mice deficient in canonical TGFb1 signaling through SMADs are protected from EoE-associated remodeling,[17] but targeting TGFβ[18] is likely difficult, given its role in multiple processes, including control of regulatory T cells. Thus, new molecules that could regulate fibroblast activity in EoE are of interest.

We previously reported that the TNF-related cytokine LIGHT (TNF superfamily member 14) has important roles in eosinophilic diseases.[19–21] LIGHT and its receptors, the herpesvirus entry mediator (HVEM) and lymphotoxin beta receptor (LTbR), contribute to fibrosis and inflammation in murine asthma and atopic dermatitis.[19–21] Moreover, LIGHT induces inflammatory mediators in lung fibroblasts.[22] LIGHT can be produced by T cells and can stimulate T-cell proliferation and cytokine production, suggesting that it will play an important role in multiple T-cell–mediated diseases.[23–26] However, although the presence of HVEM has been reported in the EoE esophagus, to our knowledge, the role of LIGHT in EoE has not been investigated.[27]

Here, we report the presence of LIGHT and its receptors in the active EoE esophagus and demonstrate the ability of LIGHT to induce a proinflammatory phenotype in fibroblasts in EoE. Moreover, cross-talk between the TGFβ1 and LIGHT pathways promotes eosinophil tethering to fibroblasts, a novel mechanism that links the fibrotic and inflammatory cascades in EoE.

Materials and Methods

Fibroblast Extraction and Culture

Human primary esophageal fibroblasts were obtained from esophageal mucosa of healthy donors (Arkansas Regional Organ Recovery Agency, Little Rock, AR) or from esophageal biopsy samples of patients with active EoE (University of California San Diego [UCSD]/ Rady Children's Hospital cohort of patients with EoE), following methods previously described.[14,28] Further information on cell extraction and maintenance can be found in the Supplementary Materials and Methods. Relevant information on the patients can be found in Supplementary Table 1. All participants provided consent/ assent to be included in the EoE database and sample collection study (UCSD/Rady Children's Hospital institutional review board no. 181690).

Reagents

A detailed list of reagents can be found in Supplementary Table 2.

Fibroblast Treatment

Fibroblasts between passages 2–5 were used. Cells were switched to basal smooth muscle cell medium without serum or supplements, containing 100 units/mL penicillin and 100 μg/ mL streptomycin 16–24 hours before treatment. For RNA sequencing (RNA-seq), healthy fibroblasts from 4 different donors were treated with vehicle, or 5 ng/mL TGFβ1, or 50 ng/ mL LIGHT for 24 hours (optimal doses identified in titration experiments). To study the effects of TGFβ1 on LIGHT receptors, fibroblasts from healthy donors or patients with active EoE were stimulated with 1 ng/mL TGFβ1 for 48 hours. To assess gene and protein expression of LIGHT targets, cells were treated with 50 ng/mL LIGHT for 24 hours. To evaluate the ability of TGFβ1 to modulate the effects of LIGHT, cells were treated with 1 ng/mL TGFβ1 for 48 hours, or were treated with 50 ng/mL LIGHT for 24 hours, or were pretreated with 1 ng/ mL TGFβ1 for 48 hours followed by 50 ng/mL LIGHT after removal

of TGFβ1. Experimental replicates were carried out on cells from different donors or patients with active EoE.

Fibroblast-Eosinophil Coculture Model

Eosinophils were purified from peripheral blood of healthy donors by negative selection (StemCell Technologies, Vancouver, Canada)[29] and pretreated with 10 ng/mL interleukin (IL) 5 for 48–72 hours. Eosinophils were then added at a 1:1 ratio to monolayers of active EoE fibroblasts cultured in 4-well slide chambers untreated or prestimulated with 50 ng/mL LIGHT for 24 hours. After 6 hours, wells were washed thoroughly to remove weakly adhered/nonadhered eosinophils. Resulting cocultures were fixed in 1% paraformaldehyde in phosphatebuffered saline for 20 minutes and stained with H&E or eosinophil peroxidase (EPX) (antibody is a kind gift from Dr Elizabeth Jacbosen).[30] To assess the involvement of intercellular adhesion molecule-1 (ICAM-1) on fibroblast-eosinophil adhesion, LIGHT-treated fibroblasts were incubated with 10 mg/mL anti–ICAM-1 antibody or isotype. After 1 hour, eosinophils were added. H&E stained cocultures were imaged using light microscopy, and the total number of eosinophils and eosinophil aggregates (>4 eosinophil per fibroblast) were counted in 4–6 high-power fields using ImageJ (Bethesda, MD). Average eosinophils or aggregates in 4–6 high-power fields were compared.

Quantitative Reverse-Transcription Polymerase Chain Reaction and Flow Cytometry

Methods, antibodies, and primers are listed in Supplementary Materials and Methods and Supplementary Tables 2 and 3.

Histology

Esophageal tissue was formalin fixed and paraffin embedded. Formalin-fixed, paraffin-embedded biopsy samples from patients with active EoE were obtained from a cohort of UCSD/Rady Children's Hospital pediatric patients. Active EoE was defined as 15 eosinophils in a high-power field. The majority of the tissues analyzed were from patients who were not receiving corticosteroid treatment. For the analysis of LIGHT and CD3, samples from 3 or 4 healthy donors and 7 or 8 patients with active EoE were stained. All specimens were suitable for epithelial quantification of LIGHT- and CD3-positive cells using the cell counter tool in Image J. Only 6 active EoE specimens had sufficient lamina propria (LP) for LIGHT quantification. Immunofluorescence for vimentin, CD90, EPX, and a– smooth muscle actin (α-SMA) was done in samples from 3 healthy and 4 or 5 patients with active EoE.

Next-Generation RNA Sequencing

Total RNA was isolated using the Qiagen miRNeasy Micro Kit (217084; Qiagen, Hilden, Germany) with optional RNase-Free DNase Set (Qiagen 79254), followed by quantification of RNA using the Qubit Fluorometer 1.0 and Qubit RNA HS Assay Kit (Q32855; Thermo Fisher Scientific, Waltham, MA). Next, 200 ng of total RNA was prepared into Illumina (San Diego, CA) messenger RNA (mRNA) libraries, according to the manufacturer's instructions, using the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB E7490L New England Biolabs, Ipswich, MA) and the NEBNext Ultra II Directional RNA Library

Prep Kit for Illumina (NEB E7760L). The resulting libraries were pooled at equimolar concentrations using Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Rockville, MD) and deep sequenced on an Illumina 2500 in Rapid Run Mode in 1 × 50 single-end configuration (approximately 25–30 million reads per condition).

RNA-Sequencing Normalization and Differential Gene Expression Analysis

Single-end reads were filtered for reads aligning to transfer RNA, ribosomal RNA, adapter sequences, and spike-in controls. Reads were aligned to GRCh38 reference genome and Gencode v27 annotations using TopHat, version 1.4.1.[31] DUST scores were calculated with PRINSEQ Lite, version 0.20.3, and lowcomplexity reads (DUST > 4) were removed from the BAM files.[32] Alignment results were parsed via SAMtools to generate SAM files.[33] Read counts to each genomic feature were obtained with the htseq-count program, version 0.7.1.[34] After removing absent features, raw counts were imported to R/Bioconductor package DESeq2, version 1.6.3, to identify differentially expressed genes among samples.[35] *P* values were calculated by using the Wald test for differences between the base means of 2 conditions and were adjusted for multiple test correction using the Benjamini-Hochberg algorithm.[36] Genes were considered differentially expressed when DESeq2 analysis showed an adjusted *P* value of <.05 and a difference in gene expression of >1.5-fold. The sequences used in this article have been submitted to the Gene Expression Omnibus under accession number GSE143482.

Single-Cell RNA-Sequencing Data Analysis

Single-cell RNA-seq expression data from EoE[37] were downloaded from the Gene Expression Omnibus under the accession number (GSE126250). We used the cluster information annotated by Wen et al[37] to investigate the expression of LIGHT. We analyzed the eight different clusters (T1-T8) identified within the $CD3^+CD45^+$ T cells recovered from patients with a spectrum of EoE disease activity (579 cells from patients with active EoE, 265 from patients with remission EOE, and 240 from control individuals).

Statistical Analysis

One-way analysis of variance in the group means was performed for multiple comparisons using the Newman-Keuls posttest. For experiments comparing only 2 groups, 2-tailed unpaired Student *t* test with a confidence level of 95% was used. Means and standard deviations were used in the study. Data were significant if *P* was <.05 (*), <.01 (**), or <.001 (***).

Results

LIGHT Is Expressed in Eosinophilic Esophagitis

To assess the relevance of LIGHT in EoE, we performed immunohistochemical analysis in esophageal biopsy samples from control individuals and patients with active EoE. $LIGHT^+$ cells were present in both the LP and epithelium of healthy and active EoE esophagi (Figure 1A). Quantitation of $LIGHT^+$ cells showed a significant increase in the epithelium of active EoE compared to healthy esophagi, and a similar trend was seen in the LP (Figure 1B and C). T cells are a likely source of LIGHT and are thought crucial for EoE pathogenesis.

Immunostaining for the T-cell marker CD3 showed abundant $CD3^+$ cells in the esophagus and a significant increase in epithelial $CD3^+$ cells in active EoE compared to healthy esophagi (Figure 1D and *E*). Interestingly, quantification of epithelial $CD3^+$ and $LIGHT^+$ cells in paired biopsy samples showed a similar significant increase in the epithelium in active EoE (Figure 1E). Furthermore, we found a positive correlation between $CD3^+$ and $LIGHT^+$ cells (Supplementary Figure 1A). To further the idea that LIGHT might derived from T cells, we analyzed a publicly available single $CD3^+$ cell RNA-seq data set.[37] Wen et al[37] identified 8 different clusters (T1-T8) among T cells from the esophagus of patients with a spectrum of EoE disease activity (579 cells from patients with active EoE, 265 from patients with remission EoE, and 240 from healthy control individuals). T1-T6 were largely $CD8^+$ cells, and T7-T8 were $CD4^+$ cells, with T7 expressing a regulatory T cell–associated phenotype. T8 consisted of 43 cells and was identified as the only bona fide effector T-helper type 2 cell population, displaying elevated IL13 and IL5 transcripts, 2 cytokines implicated in EoE disease development. Overall, 42 out of 43 T8 cells were found in active EoE, and only 1 T8 cell was found in remission. We found that $LIGHT^+$ cells were present in all of the different T-cell clusters in patients with EoE (active and remission combined), with the putative pathogenic T8 population showing a significantly higher expression compared to the other clusters, with the exception of T5, which consisted of $CD8^+$ interferon gamma–expressing cells (Wilcoxon rank sum test, $P < .05$) (Figure 1F). Analysis of LIGHT transcripts in only the LIGHT-expressing cells showed a similar level in all clusters (Supplementary Figure 1B), suggesting that the primary difference between clusters was in the number of LIGHT-expressing cells. In line with this, a comparison of the total number of T cells ($CD3^+$) and the number of LIGHT-expressing T cells ($LIGHT^+$) showed parallel increases in each cluster during active EoE, with the exception of T1, and a reduction to levels seen in normal individuals during EoE remission (Figure 1G and H). These data align with our histologic analysis that showed significant increases in $CD3^+$ and $LIGHT^+$ cells during active EoE (Figure 1A–E). Analysis of the proportion of $LIGHT^+$ cells per cluster showed that the T8 cluster had the highest proportion of $LIGHT^+$ cells in active EoE and a general decrease in the proportion of $LIGHT^+$ cells in many of the clusters during remission (Supplementary Figure 1C and D). Consistent with this, $LIGHT^+$ T cells in all clusters together represented 43.9% of the total T cells during active EoE and 31.7% during remission (Supplementary Figure 1E). A comparative analysis showed that LIGHT mRNA transcript levels in T8 cells were equivalent to those of IL5 and IL13 (Figure 1H). These data show that $LIGHT^+$ cells are present in the esophagus and increase in number during active EoE.

LIGHT Induces a Unique Inflammatory Transcriptomic Profile in Esophageal Fibroblasts

To understand one potential target cell responsive to LIGHT in EoE, we analyzed the expression of its receptors in esophageal fibroblasts. HVEM and LTβR were expressed on normal and active EoE fibroblasts at similar levels (Figure 2A). To investigate the transcriptome induced by LIGHT and compare it to that induced by the profibrotic cytokine TGFβ1, we treated esophageal fibroblasts from 4 healthy individuals with LIGHT or TGFβ1 for 24 hours and performed RNA-seq. LIGHT predominantly induced proinflammatory pathways including TNF or TNF family molecule signaling, canonical and noncanonical nuclear factor (NF) κB activation (Table 1). Database for Annotation, Visualization, and

Integrated Discovery (DAVID) analysis showed significant enrichment in 4 major pathways: (1) TNF/NF-κB signaling pathway (enrichment score [ES], 2.94), (2) NK-KB Inducing Kinase/NF- κ B signaling pathway (ES, 2.26), (3) regulation of the NF- κB signaling pathway (ES, 2.11), and (4) regulation of the TNF-mediated signaling pathway (ES, 1.59). The transcripts up-regulated by LIGHT were largely nonoverlapping with those induced by TGFβ1 and included interleukins (IL32, IL33, IL34) and interleukin receptors (IL4R and IL27RA), CD molecules (CD74, CD82), TNF superfamily receptors or cytokines (TNFRSF1B, TNFSF13B, TNFSF15), chemokines and growth factors (CXCL5, CSF1), complement factors (C3), and adhesion molecules (ICAM-1) (Supplementary Figure 2A). TGFβ1 regulated the largest cohort of mRNAs, modifying the pattern of expression of approximately 7000 genes and showing almost equal activity for down- (3713 genes) and up-regulation of gene expression (3556 genes) (Figure 2B and Supplementary Figure 2B). In contrast, LIGHT influenced fewer transcripts and acted predominantly as a transcriptional activator, inducing 472 and repressing 131 genes (Figure 2B and Supplementary Figure 2C). Moreover, LIGHT had a greater number of uniquely up-regulated targets than those it shared with TGFβ1 (367 vs 105) (Figure 2B). The common up-regulated transcripts induced by TGFβ1 and LIGHT are detailed in Supplementary Table 4.

TGFβ1 is a key regulator of fibrotic responses in fibroblasts, principally through promoting differentiation into myofibroblasts. We assessed markers of myofibroblast differentiation compared to inflammatory-type genes after LIGHT or TGFβ1 treatment. Although TGFβ1 induced a myofibroblast phenotype, LIGHT induced a distinct inflammatory phenotype without associated myofibroblast markers (Figure 2C). These phenotypes aligned accurately to the myofibroblast and inflammatory fibroblast phenotypes previously described in the colon.[9] For example, LIGHT up-regulated expression levels of CD74, ICAM-1, IL-32, IL33, IL34, C3, RBP5, TNFSF13B, and IRF8, whereas TGFb1 had little/no effect on these (Figure 2C). In contrast, TGFβ1 up-regulated α-SMA (ACTA2), myosin heavy chain (MYH11), collagen (COL1A1), and other smooth muscle-associated myofibroblast molecules (TAGLN, FLNA, and ACTG2), as well as fibrosis mediators PLN, CTGF, or SERPINE1 (Figure 2C). The latter was also induced by LIGHT, but TGFβ had a far greater effect (Supplementary Table 4).

Validation of subsets of proinflammatory molecules using quantitative reverse-transcription polymerase chain reaction (qRT-PCR) in fibroblasts from different healthy donors and patients with active EoE proved the integrity of the RNA-seq data set (Figure 2D–I). This also showed significant differences in the response between normal and active EoE fibroblasts for certain genes, although enhanced responsiveness was not universally seen. For example, active EoE fibroblasts had higher baseline and LIGHTinduced IL32 and ICAM-1 transcripts than normal fibroblasts (Figure 2D and E) but lower IL33 (Figure 2H). These data suggest that TGFβ1 and LIGHT have nonredundant functions and may induce differentiation into distinct populations of pathologically relevant esophageal fibroblasts.

Transforming Growth Factor β1 Increases Herpesvirus Entry Mediator Expression and Modulates LIGHT-Induced Gene Expression in Esophageal Fibroblasts

We next asked whether TGFβ1 and LIGHT would exhibit cross-talk that could further modulate the differentiation state of esophageal fibroblasts and if TGFβ1 could affect the expression of the receptors for LIGHT. TGFβ1 exposure significantly increased the expression of HVEM, but not LTβR, in normal and EoE fibroblasts (Figure 3A and B). Based on this, we pretreated fibroblasts with TGFβ1 followed by exposure to LIGHT. This resulted in a complex and new fibroblast phenotype with further up-regulation of some inflammatory transcripts that were induced by LIGHT treatment alone, but down-regulation of others (Figure 3C and D). Among the tested targets, there was a differential effect on the adhesion molecules, ICAM-1 and VCAM-1. Although ICAM-1 was more greatly up-regulated by LIGHT after TGFβ1 pre-exposure, VCAM-1 expression induced by LIGHT was inhibited by pre-exposure to TGFβ1 (Figure 3C and D). In keeping with this, similar effects of pre-exposure to TGFβ1 on the activity of LIGHT were seen for other targets. IL32, IRF8, CCL5, or IL6 were more significantly upregulated by LIGHT after TGFβ1 pre-exposure; BIRC3 showed no major changes; and CD74, CX3CL1, IL33, IL34, and CXCL5 were not induced or were less strongly induced by LIGHT after TGFβ1 pre-exposure. In contrast, the myofibroblast phenotype driven by TGFβ1 was largely unchanged by subsequent LIGHT stimulation (Figure 4E). These variable changes implied they were not simply a result of enhanced HVEM expression induced by TGFβ1 and greater signaling by LIGHT. Thus, TGFβ1 cross-talk with LIGHT resulted in a myofibroblast-like population that upregulated select inflammatory molecules, resulting in a phenotype that was a combination of a LIGHT-driven and a TGFβ1–driven cell.

We then focused on the expression of adhesion molecules in relation to α-SMA in fibroblasts from patients with active EoE to further investigate these populations. Flow cytometry confirmed that sequential TGFβ1 and LIGHT treatment led to greater ICAM-1 expression than with LIGHT stimulation alone, whereas VCAM-1 was upregulated by LIGHT alone but negated by prior TGFβ1 exposure (Figure 4A and B). EoE fibroblasts had little α-SMA, and LIGHT treatment did not affect its expression (Figure 4C, LIGHT), whereas consecutive treatment with TGFβ1 and LIGHT resulted in an ICAM-1^{high}α-SMA^{high} phenotype (Figure 4C, TGFβ1 + LIGHT). Immunofluorescence staining of TGFβ1–pretreated cells stimulated with LIGHT confirmed the presence of ICAM-1^{+}α-SMA^{+} double-positive cells (Figure 4D). VCAM-1 was present in esophageal fibroblasts in basal conditions and upregulated by LIGHT, but sequential TGFβ1 and LIGHT treatment promoted both VCAM-1^{high}a-SMA^{high} and VCAM-1^{low}a-SMA^{high} populations (Figure 4E, LIGHT vs TGFβ1 + LIGHT). The majority of these VCAM-1^{high} and VCAM-1^{low}a-SMA^{high} populations were ICAM-1^{high} (Figure 4F). These data suggest the existence of a mixed inflammatory myofibroblast phenotype promoted by the crosstalk with LIGHT.

Fibroblasts Interact With Eosinophils in Active Eosinophilic Esophagitis

Given the ability of fibroblasts to up-regulate inflammatory genes involved in eosinophilic inflammation, we next investigated the potential colocalization of fibroblasts and eosinophils in EoE.[38–42] CD90^{+} and vimentin^{+} commonly used fibroblast markers,[9,10,43] were highly expressed in fibroblasts from healthy and active EoE esophagi (Figure 5A and B). Although

treatment with either LIGHT or TGFb1 increased ICAM-1 and α-SMA, respectively, neither CD90 or vimentin expression was altered (Figure 5A and B). Double immunofluorescence staining confirmed the co-expression of CD90-vimentin in cells localized to the LP of healthy esophagi (Figure 5C).

Interestingly, in patients with active EoE, CD90^{+}·vimentin^{+} cells were not restricted to the LP but also infiltrated the epithelium (Figure 5C). Analysis of a-SMA as a myofibroblast marker showed that vimentin^{+}/α-SMA^{+} fibroblasts represented a minority of fibroblasts and that these were mainly localized to the LP in both healthy and active EoE biopsy samples (Figure 5D). Finally, staining for the specific eosinophil marker EPX showed an abundance of EPX^{+} eosinophils in the LP and epithelium of patients with active EoE (Figure 5E). Most importantly, vimentin^{+} fibroblasts were often found in close proximity to eosinophils in the LP and epithelium (Figure 5E). This suggests a potential contribution of fibroblasts to eosinophilic inflammation in EoE.

LIGHT Mediates Fibroblast-Eosinophil Interactions via Intercellular Adhesion Molecule-1

An ingenuity pathway analysis predicted an enrichment of immune cell trafficking activities in LIGHT-treated esophageal fibroblasts (Supplementary Table 5). Consistent with the hypothesis that LIGHT exposure of fibroblasts might be linked with eosinophilia, the percentage of ICAM1^{high}, but not α-SMA^{high}, fibroblasts derived from esophageal biopsy samples correlated directly with the number of eosinophils per high-power field in patients with active EoE (Figure 6A and B). We then asked if EoE esophageal fibroblasts could adhere to human eosinophils and if this was regulated by LIGHT. An eosinophil-fibroblast coculture model showed low-level binding without fibroblast stimulation but that LIGHT treatment strongly enhanced the adherence of multiple eosinophils, often in aggregates (Figure 6C). Moreover, it appeared that LIGHT-stimulated fibroblasts were activating eosinophils because extracellular EPX was present in the adherent cells (Figure 6C). Furthermore, anti–ICAM-1 treatment reduced the number of eosinophils and eosinophil clusters adhered to LIGHT-treated fibroblasts to levels seen with nonstimulated fibroblasts (Figure 6D and E). Thus, LIGHT-stimulated esophageal fibroblasts convert to an inflammatory phenotype that promotes the tethering of eosinophils via expression of ICAM-1.

Discussion

Here, we document a number of novel findings in EoE. We show the presence of LIGHT^{+} cells in the LP and epithelium in the esophagus and an increase in the number of LIGHT^{+} cells during active EoE. We show that mRNA for LIGHT is expressed in several different esophageal T-cell populations, including the majority of IL13^{+}IL5^{+}CD4^{+} T cells in active EoE. We show that LIGHT promotes a unique inflammatory gene signature in esophageal fibroblasts and modifies TGF-β–driven myofibroblast differentiation to promote a distinct inflammatory type of myofibroblast. Finally, we document that one consequence of LIGHT-induced inflammatory activity is the accumulation and localization of eosinophils through an ICAM-1-dependent interaction with esophageal fibroblasts.

LIGHT is a TNF superfamily protein that contributes to inflammation and fibrosis in several animal models of disease.[19–21,44] LIGHT inhibition or deficiency protects mice from asthmatic airway remodeling or skin inflammation, and correspondingly, intratracheal or subcutaneous injection of recombinant LIGHT causes pulmonary or skin fibrosis.[19–21] LIGHT may act on numerous cell types that express its receptors and may drive remodeling and inflammatory responses through acting on macrophages, eosinophils, fibroblasts, and epithelial cells.[21,22,45,46] Our data now show that LIGHT promotes proinflammatory gene expression in esophageal fibroblasts that is likely relevant to the development of esophageal inflammation and remodeling. Notably, the LIGHT transcriptome profile is distinct from that induced by TGFβ1, indicating nonredundant functions of .IGHT in esophageal fibroblasts.

A number of recent studies have provided insight into different populations of fibroblasts that may vary over the course of disease development.[9–11] Most notably with regard to our findings, single-cell gene expression showed an inflammatory type of fibroblast in the colon that was expanded in patients with ulcerative colitis.[9] LIGHT treatment of esophageal fibroblasts induced many of the genes found in these colonic inflammatory fibroblasts, including *ICAM1, CD74, IL32, IL33, C3, RBP5, TNFSF13B*, and *IRF8* (Figure 3E). A second subset of fibroblasts present in the inflamed colon included myofibroblasts.[9] In our studies, TGFb1 up-regulated genes in esophageal fibroblasts that aligned with the ones found in colonic myofibroblasts in ulcerative colitis, including *ACTA2, MYH11, TAGLN, FLNA,* and *ACTG2*. Because we now show that LIGHT is present in the EoE esophagus, our studies of LIGHT likely show populations of fibroblasts that will naturally develop in EoE.[12–15] This is supported by the rather remarkable correlation of ICAM-1 positive esophageal fibroblasts in vitro with the severity of in vivo eosinophil accumulation and the fact that after in vitro culture under neutral conditions, most fibroblasts had an inflammatory rather than myofibroblast phenotype based on ICAM-1, VCAM-1, and a-SMA expression (Figure 5).

Many different cytokines are likely to be simultaneously present in the EoE esophagus, and their relative amounts may exert a concerted action on disease pathogenesis. Although TGFb1 promotes wound healing, its continued expression in EoE is thought to be a driver of pathologic and, at times, irreversible remodeling. TGFβ1 is produced by several inflammatory cells in EoE and is abundant in the inflamed EoE esophagus, and its polymorphisms can exacerbate fibrosis.[13,47] Our new data suggest that LIGHT is also abundant in the EoE esophagus, suggesting it may integrate with TGFb1 to promote pathology. Correspondingly, we found that TGFβ1 modulated LIGHT-induced gene expression, and the resulting fibroblasts displayed a novel phenotype, to our knowledge, that was both myofibroblast and inflammatory. Although TGFβ1 increased the expression of the LIGHT receptor HVEM, it is unlikely that only signals from HVEM were important to this resultant phenotype. For example, our studies previously showed that LTβR was central to the production of a number of inflammatory mediators in lung fibroblasts.[22] In contrast, both HVEM and LTβR were critical for keratinocyte proliferation, whereas expression of TSLP and periostin by these cells was solely dependent on HVEM.[21] Further studies are warranted to understand the contribution of both LIGHT receptors to esophageal inflammatory fibroblasts and how signaling from these receptors is regulated by TGFβ1. Moreover, it will

be interesting in future studies to understand if this novel fibroblast phenotype is present in the EoE esophagus directly ex vivo and whether it corresponds to the stage of disease when fibrosis occurs. In this context, TGFb1 activity could promote tissue remodeling indirectly by enhancing ICAM-1 expression on fibroblasts driven by LIGHT, leading to increased eosinophil accumulation and retention at the sites of fibrosis.

The source of LIGHT in EoE still requires further study. LIGHT is expressed in T cells in various tissues, and we now show that LIGHT is made by T cells in the esophagus. Constitutive LIGHT expression in T cells has been shown to cause inflammation and tissue destruction in mice,[23,48] implying that T cell–derived LIGHT is likely important. However, other cell types are capable of expressing LIGHT, including neutrophils, dendritic cells, natural killer cells, and macrophages, and colonic inflammatory fibroblasts were also found to express abundant LIGHT transcripts.[9,24,49] Therefore, although T cells are most likely a primary source of LIGHT, we cannot rule out the possibility that other cell types may contribute LIGHT in EoE. LIGHT has been implicated in the recruitment, maturation, activation, and/or adhesion of different immune cells, including T cells, eosinophils, basophils, mast cells, and dendritic cells.[26,50–52] LIGHT mediated the expression of adhesion molecules and the adhesion of eosinophils or basophils and bronchial epithelial cells.[51] In line with this, our analysis of the LIGHT transcriptome in esophageal fibroblasts showed enrichment of immune cell trafficking. Together with immunostaining showing colocalization of fibroblasts and eosinophils in active EoE, as well as coculture of LIGHT-treated esophageal fibroblasts resulting in increased eosinophil tethering and, potentially, activation, our study suggests several novel therapeutic targets in EoE. These could include LIGHT itself. Alternatively, because blockade of ICAM-1 reduced the number of eosinophils adhering to LIGHT-treated fibroblasts, anti–ICAM-1 antibodies may be of therapeutic benefit in EoE. Similar effects have been reported for blockade of the ICAM-1–dependent interaction between T cells and endothelial cells,[53] and ICAM-1 has been targeted to reduce T-cell adhesion to intestinal fibroblasts.[39]

In summary, we show that LIGHT is a pathogenic cytokine expressed in T cells in EoE that drives a proinflammatory esophageal fibroblast phenotype. Although many patients with EoE respond to anti-inflammatory therapy, a subset does not have adequate inflammatory control with standard therapies. For these individuals, there are limited options. Our results warrant future studies to further elucidate the role of LIGHT in EoE, including in specific subpopulations of patients to understand if they might benefit from LIGHT-blocking therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper:

DAVID	Database for Annotation, Visualization, and Integrated Discovery
EoE	eosinophilic esophagitis
EPX	eosinophil peroxidase
ES	enrichment score
HVEM	herpesvirus entry mediator
ICAM-1	intercellular adhesion molecule-1
IL	interleukin
LP	lamina propria
LTβR	lymphotoxin beta receptor
mRNA	messenger RNA
NF	nuclear factor
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
RNA-seq	RNA sequencing
SMA	smooth muscle actin
TGFb1	transforming growth factor-beta
TNF	tumor necrosis factor
TNFSF14/LIGHT	tumor necrosis factor superfamily 14
UCSD	University of California, San Diego
VCAM-1	vascular cell adhesion molecule-1

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WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Eosinophilic esophagitis (EoE) involves fibroblast activation and progression to fibrostenosis. This study investigated cytokines and cell types that contribute to EoE development.

NEW FINDINGS

T cells in esophageal tissues from patients with EoE express increased levels of LIGHT, which caused esophageal fibroblasts to acquire an inflammatory phenotype. TGFβ1 increased fibroblast expression of HVEM, a receptor for LIGHT. Fibroblasts incubated with LIGHT bound to eosinophils in vitro via ICAM1.

LIMITATIONS

This study was performed using human tissues and cells; further studies are needed to determine the mechanisms of this process.

IMPACT

Strategies to target this pathway might be developed for treatment of EoE.

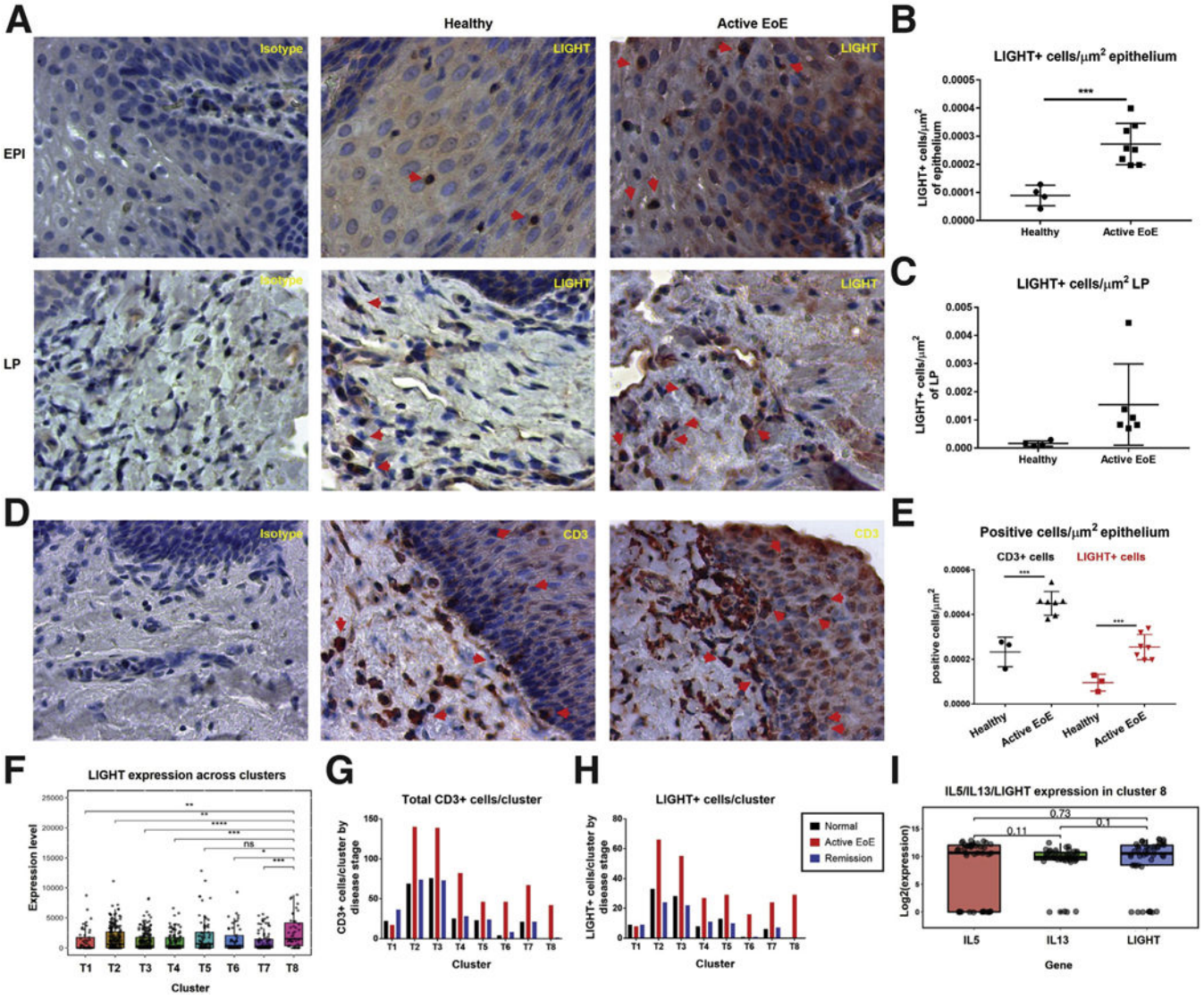

Figure 1.

LIGHT is expressed in active EoE. (*A*) Representative image of LIGHT$^+$ cells in the epithelium (EPI) (*top*) and LP (*bottom*). Quantification of LIGHT-positive cells/μm^2 in the (*B*) epithelium and (*C*) LP of biopsy samples from 4 normal and 6 to 8 active EoE esophagi. (*D*) Representative images of CD3$^+$ cells in biopsy samples from 4 normal and 7 active EoE esophagi. (*E*) Quantification of epithelial CD3$^+$ and LIGHT$^+$ cells in paired biopsy samples from healthy individuals and patients with active EoE. Each dot represents an independent donor (healthy) or patient (Active EoE). Arrows point to LIGHT or CD3 stained cells. (*F*) LIGHT transcript abundance in 8 clusters of esophageal T cells (T1–T8) defined by Wen et al.[37] (G) Total number of CD3$^+$ cells and (H) total number of LIGHT$^+$ cells in each T-cell cluster comparing cells from healthy, active EoE, or remission. (*I*) Comparative analysis of IL5, IL13, and LIGHT expression in T8 cells. **P* < .05, ***P* < .01, ****P* < .001.

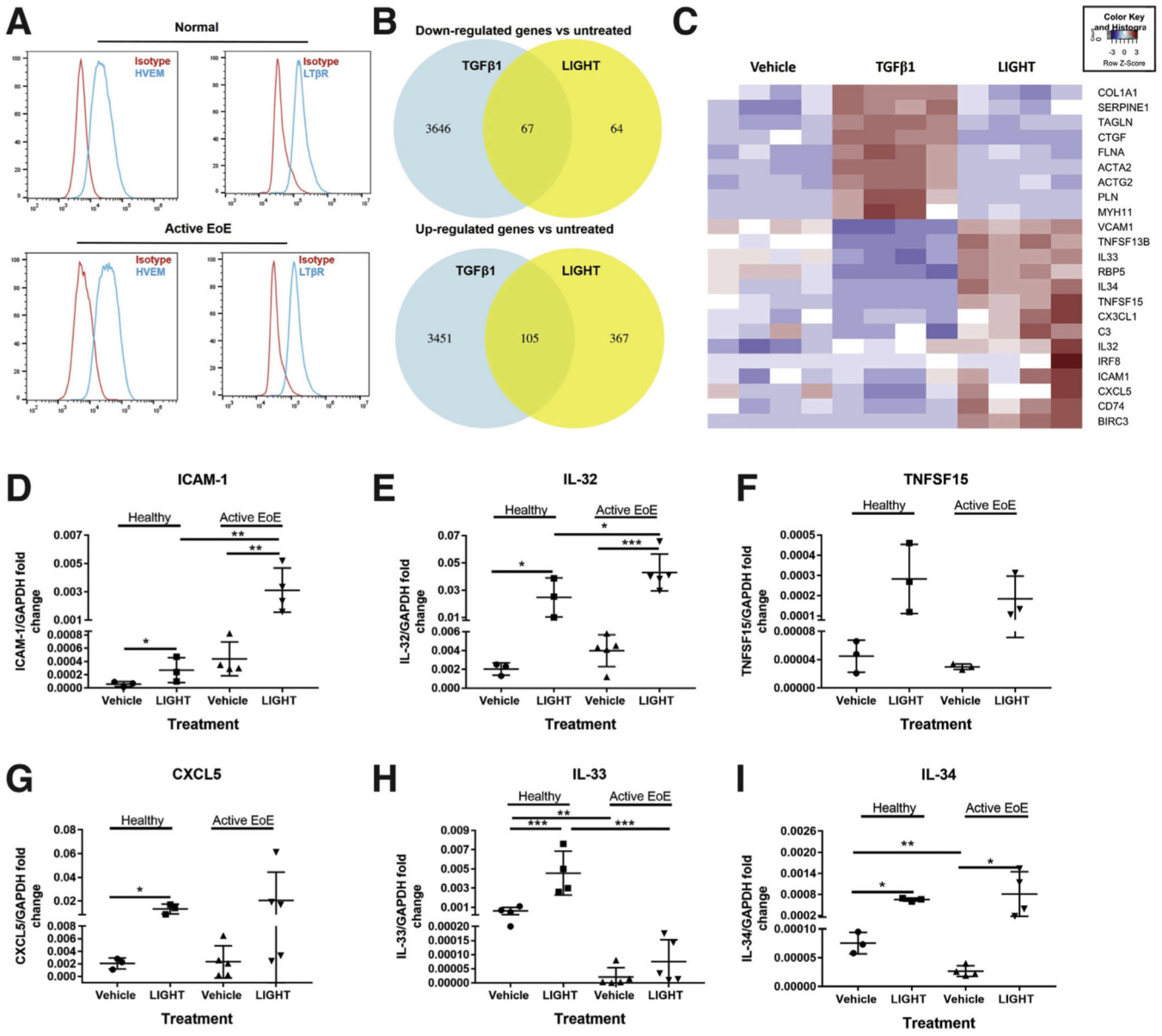

Figure 2.

LIGHT induces a unique proinflammatory gene expression profile in esophageal fibroblasts. (*A*) Representative flow cytometry analysis of baseline HVEM and LTbR in healthy (n = 4) or active EoE (n = 5) esophageal fibroblasts. (*B*) Venn diagram of genes down-regulated (*top*) or up-regulated (*bottom*) by TGFβ1 and LIGHT compared to untreated in healthy esophageal fibroblasts (n = 4; *P* adj < .05; >1.5-fold). (*C*) Heatmap comparing the expression levels of genes associated with myofibroblast or inflammatory phenotype in esophageal fibroblasts treated with TGFβ1 or LIGHT (n = 4; *P* adj < .05; >1.5fold). (*D–I*) qRT-PCR analysis of some LIGHT-induced targets in healthy and active EoE fibroblasts. Each dot represents an independent donor (healthy, n = 3) or patient (Active EoE, n = 3–5). Differences are significant if **P* < .05, ***P* < .01, or ****P* < .001.

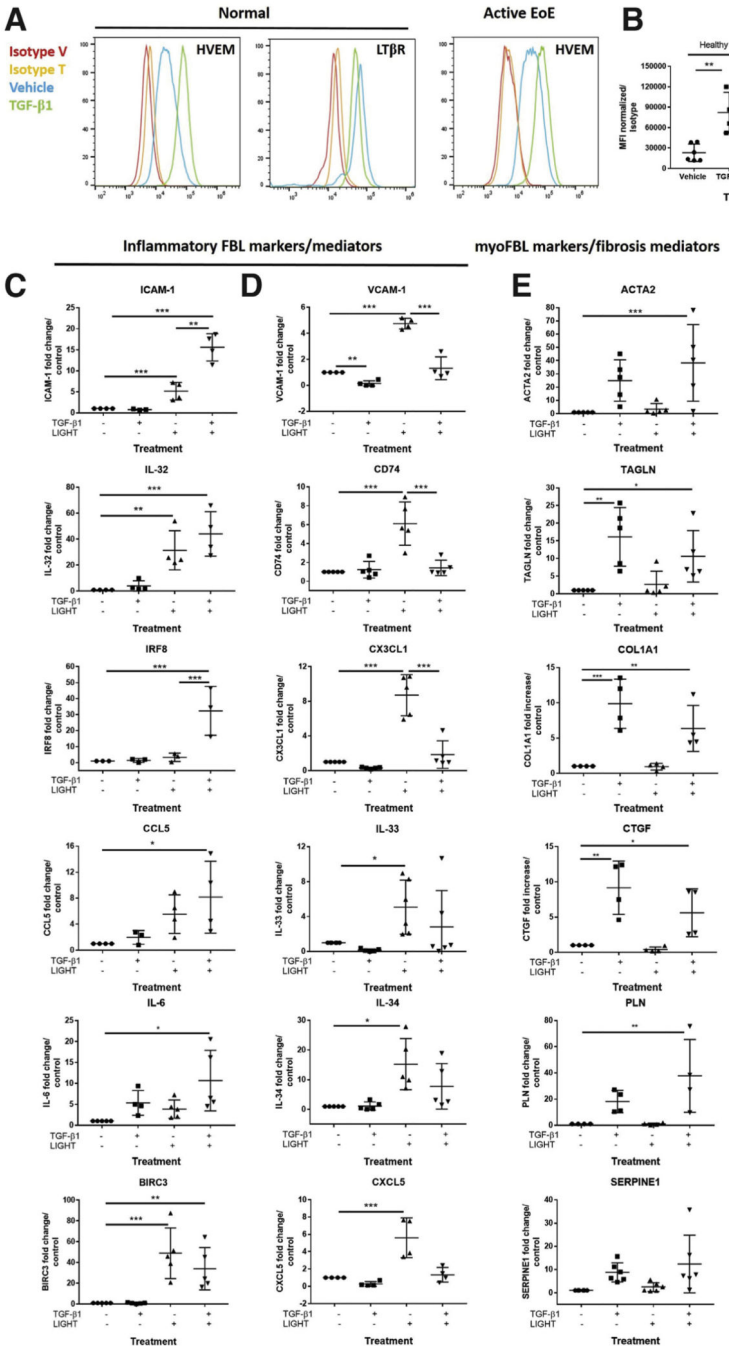

Figure 3.

TGFβ1 increases HVEM expression and modulates LIGHT-induced gene expression in esophageal fibroblasts. (*A*) Representative flow cytometry histograms of HVEM and LTβR in esophageal fibroblasts from healthy donors or patients with active EoE untreated or treated with TGFβ1 for 48 hours. (*B*) Mean fluorescence intensity of HVEM in normal (n = 6) or active EoE (n = 10) fibroblasts. (*C–E*) qRT-PCR of (*C, D*) inflammatory molecules (ICAM-1, IL32, IRF8, CCL5, IL6, BIRC3, VCAM-1, CD74, CX3CL1, IL-33, IL-34 and CXCL5) and (*E*) myofibroblast and fibrosis markers (ACTA2, TAGLN, COL1A1, CTGF,

PLN, and SERPINE1) in esophageal fibroblasts from healthy donors sequentially treated with TGFβ1 for 48 hours and LIGHT for 24 hours. n = 3–5; each dot represents an independent donor. Differences are significant if *$P < .05$, **$P < .01$, or ***$P < .001$.

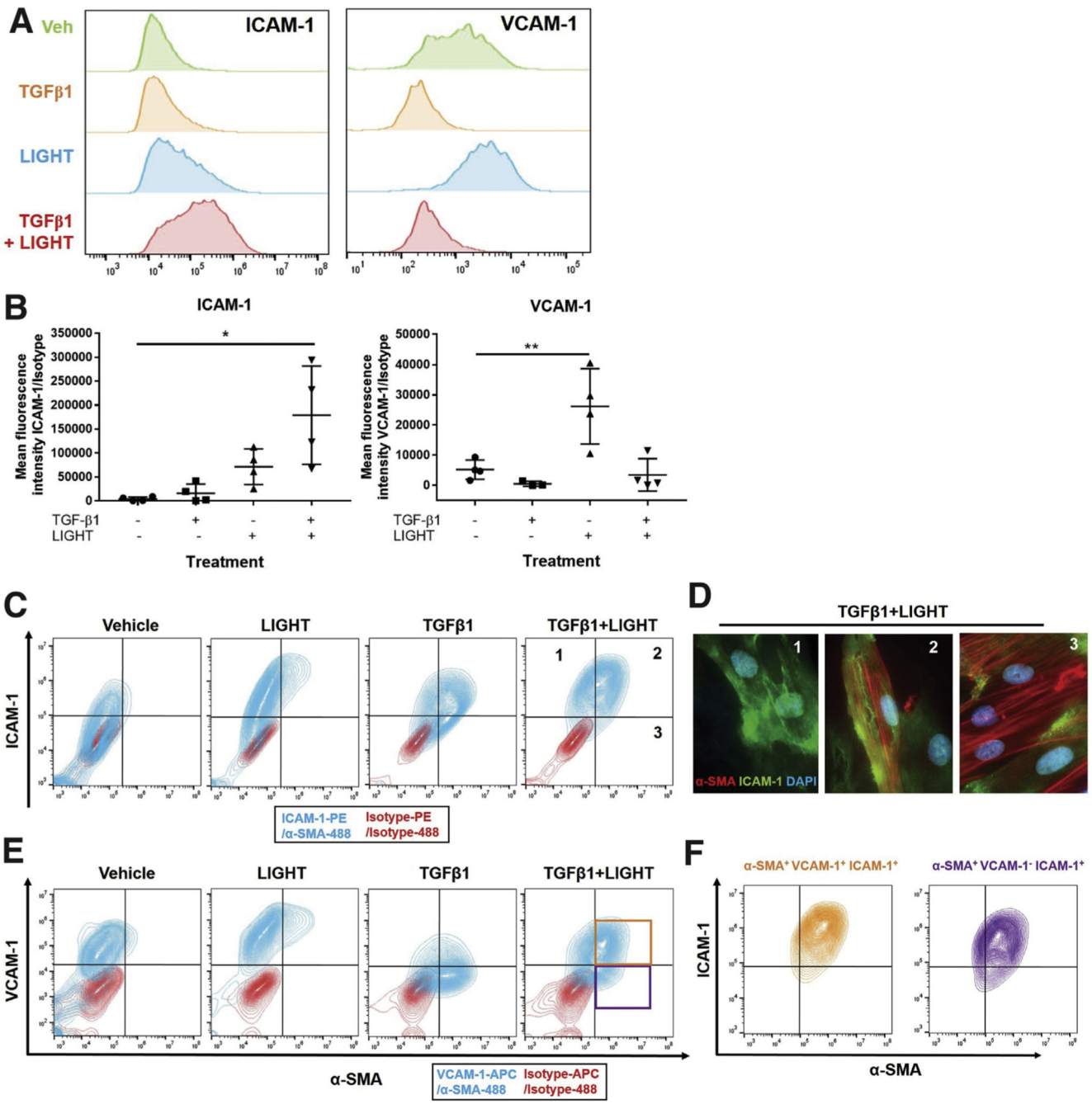

Figure 4.

Cross-talk between TGFβ1 and LIGHT modulates adhesion molecule expression in esophageal fibroblasts. (*A*) Representative histograms and (*B*) mean fluorescence intensity of ICAM-1 and VCAM-1 analyzed by flow cytometry in active EoE fibroblasts treated with TGFβ1 for 48 hours or LIGHT for 24 hours, or sequentially treated with TGFβ1 and LIGHT as in Figure 4 (n = 5). Each dot represents cells from an independent patient; *$P < .05$. (*C*) Representative population analysis of α-SMA and ICAM-1 in fibroblasts from patients with active EoE treated as in *A* and *B* (n = 13). (*D*) Representative immunofluorescence staining

of fibroblasts from patients with active EoE sequentially treated with TGFβ1 and LIGHT (n = 3). (*E*) Representative population analysis of α-SMA and VCAM-1 in fibroblasts from patients with active EoE treated as in *A* and *B*. (*F*) Representative population analysis of ICAM-1 expression within the VCAM-1$^{low}\alpha$-SMA^{high} and VCAM-1$^{high}\alpha$-SMA^{high} fibroblast populations identified in *E* (n = 13). Differences are significant if $*P < .05$.

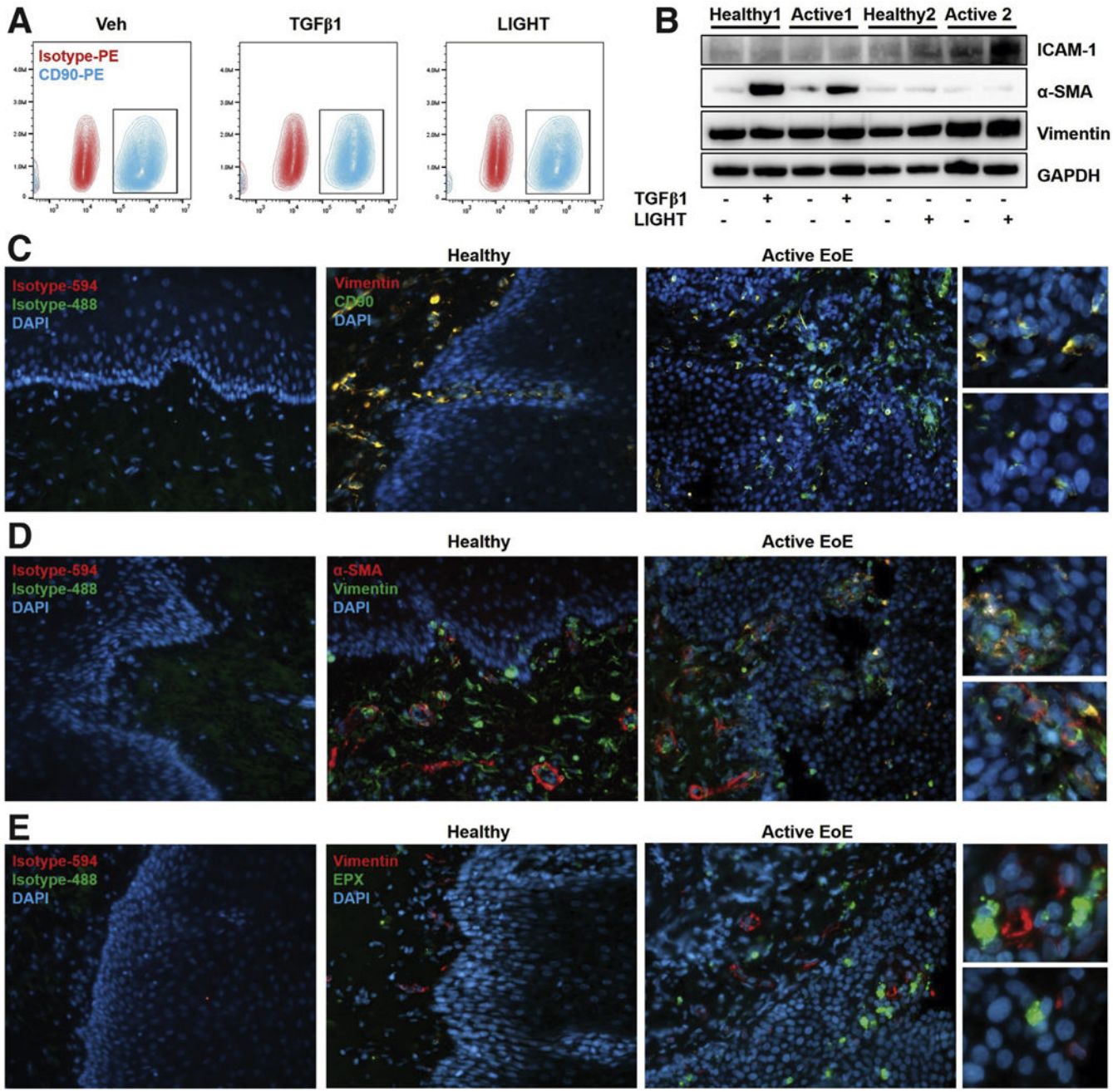

Figure 5.

Fibroblasts interact with activated eosinophils in the EoE esophagus. (*A*) representative contour plots of CD90 in active EoE fibroblasts untreated, or treated with 1 ng/mL TGFβ1, or treated with 50 ng/mL LIGHT. (*B*) Western blot analysis of vimentin, α-SMA, and ICAM-1 in fibroblasts derived from 2 healthy and 2 active EoE esophageal biopsy samples treated as in *A*. Representative images of healthy (n = 3) and active EoE (n = 4) esophageal biopsy samples double-labeled for (*C*) CD90/ vimentin, (*D*) vimentin/α-SMA, and (*E*) vimentin/EPX or their corresponding isotypes and imaged by using a fluorescence microscope.

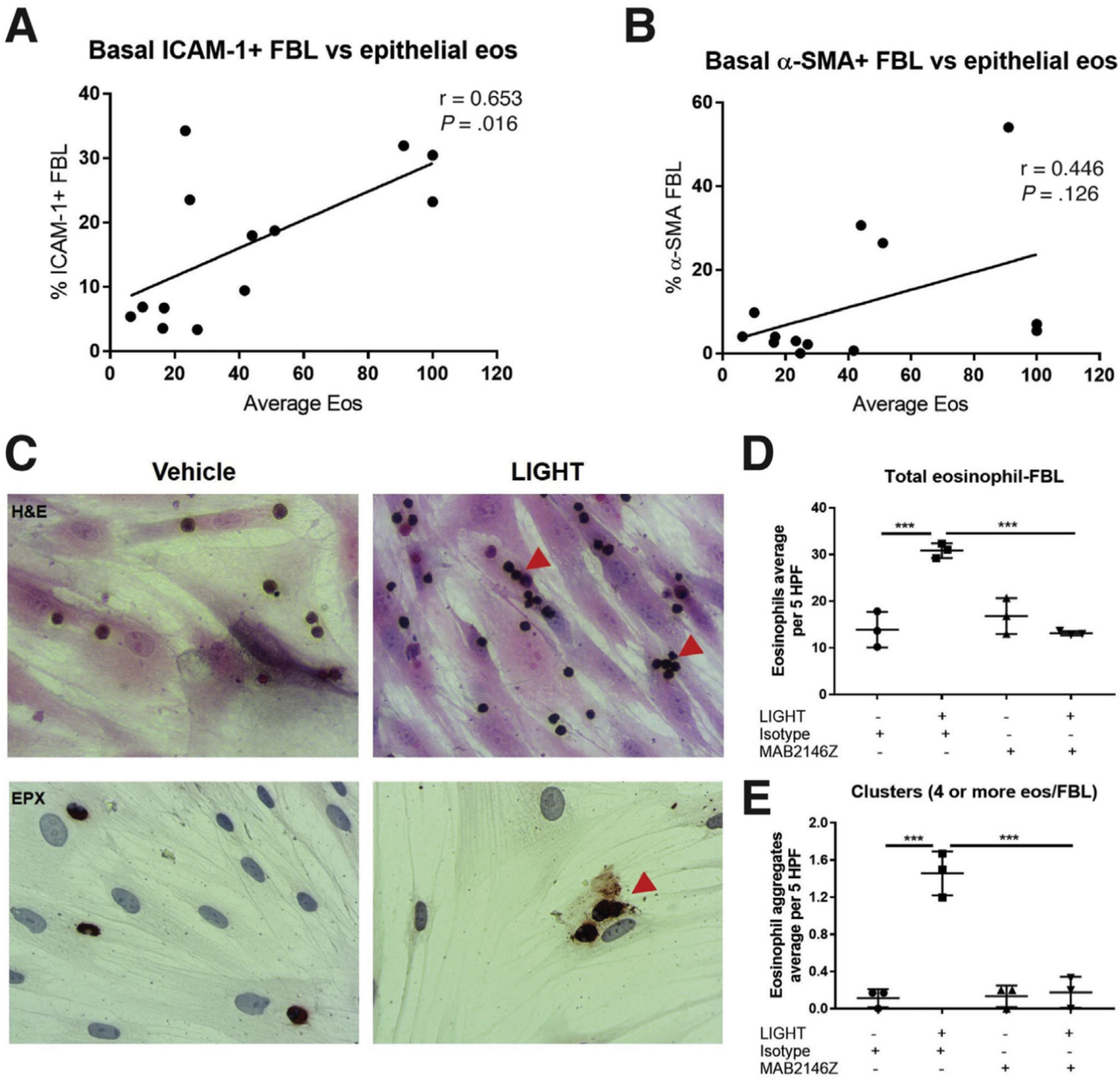

Figure 6.

LIGHT mediates fibroblast-eosinophil interactions via ICAM-1. (*A, B*) Correlation of eosinophil counts in esophageal biopsy samples with (*A*) ICAM-1^{+} or (*B*) *α*-SMA $^{+}$fibroblasts derived in vitro from the biopsy samples. Each dot represents data from an independent patient with active EoE (n = 13, Pearson's correlation coefficient). (*C*) Representative images of H&E- (n = 8) or EPX- (n = 2) stained cocultures of active EoE esophageal fibroblasts treated with LIGHT for 24 hours, then washed and cocultured with IL5-prestimulated peripheral blood eosinophils for 6 hours. Red arrows point to eosinophil aggregates on a single fibroblast. Quantification of (*D*) total numbers of eosinophils or (*E*) eosinophil aggregates (≥4 eosinophils/fibroblast) in active EoE fibroblasts unstimulated or

stimulated with 50 ng/mL LIGHT for 24 hours followed by incubation with 10 μg/mL isotype or anti–ICAM-1 antibody (MAB2146Z) and cocultured with eosinophils for 6 hours (n = 3). Quantifications show the average number of eosinophils or clusters in 6 high-power 20× fields in independent experiments with cells from different patients/donors. Differences are significant if $*P < .05$ or $***P < .001$. eos, eosinophils.

Table 1.

Functional Clustering (DAVID) of the Enriched Gene Sets[a]

Pathway ID	Pathway name	Count	%	List total	Pop hits	Pop total	Fold enrichment	*P* value
Enrichment score: 2.94								
h_tnfr2Pathway	TNFR2 signaling pathway	5	5.56	18	17	1566	25.59	2.08E–05
h_tall1Pathway	TACI and BCMA stimulation of B-cell immune responses	4	4.44	18	14	1566	24.86	3.60E–04
h_cd40Pathway	CD40L signaling pathway	4	4.44	18	15	1566	23.20	4.47E–04
h_deathPathway	Induction of apoptosis through DR3 and DR4/5 death receptors	4	4.44	18	33	1566	10.55	4.74E–03
h_keratinocytePathway	Keratinocyte differentiation	4	4.44	18	45	1566	7.73	1.14E–02
h_nfkbPathway	NF-κB signaling pathway	3	3.33	18	21	1566	12.43	2.06E–02
h_stressPathway	TNF/stress-related signaling	3	3.33	18	25	1566	10.44	2.87E–02
G0_0007249	I-κB kinase/NF-κB signaling	5	5.56	74	59	15,794	18.09	1.58E–04
G0_0033209	TNF-mediated signaling pathway	5	5.56	74	112	15,794	9.53	1.79E–03
G0_0016567	Protein ubiquitination	6	6.67	74	345	15,794	3.71	2.17E–02
G0_0005164	TNF receptor binding	3	3.33	77	29	15,898	21.36	8.42E–03
G0_0016874	Ligase activity	5	5.56	77	264	15,898	3.91	3.76E–02
hsa04064	NF-κB signaling pathway	10	11.11	44	83	6485	17.76	2.35E–09
hsa04668	TNF signaling pathway	9	10.00	44	105	6485	12.63	3.28E–07
hsa04380	Osteoclast differentiation	9	10.00	44	129	6485	10.28	1.59E–06
hsa04210	Apoptosis	4	4.44	44	60	6485	9.83	7.15E–03
R-HSA-5676594	TNFSF members mediating noncanonical NF-κB pathway	5	5.56	49	16	8638	55.09	1.45E–06
R-HSA-5668541	TNFR2 noncanonical NF-κB pathway	5	5.56	49	64	8638	13.77	4.18E–04
Enrichment score: 2.26								
G0_0007249	I-κB kinase/NF-κB signaling	5	5.56	74	59	15,794	18.09	1.58E–04
GO_0038061	NIK/NF-κB signaling	4	4.44	74	65	15,794	13.13	3.37E–03
hsa04010	MAPK signaling pathway	7	7.78	44	250	6485	4.13	5.68E–03
R-HSA-5676590	NIK→noncanonical NF-κB signaling	3	3.33	49	57	8638	9.28	3.98E–02
R-HSA-5607761	Dectin-1 mediated noncanonical NF-κB signaling	3	3.33	49	58	8638	9.12E	4.10E–02
Enrichment score: 2.11								
h_tnfr2Pathway	TNFR2 signaling pathway	5	5.56	18	17	1566	25.59	2.08E–05
GO_0051092	Positive regulation of NF-κB TF activity	5	5.56	74	126	15,794	8.47	2.75E–03

Pathway ID	Pathway name	Count	%	List total	Pop hits	Pop total	Fold enrichment	*P* value
GO_0032088	Negative regulation of NF-κB TF activity	4	4.44	74	67	15,794	12.74	3.67E–03
R-HSA-933542	TRAF6 mediated NF-κB activation	3	3.33	49	23	8638	22.99	7.10E–03
hsa04622	RIG-I-like receptor signaling pathway	4	4.44	44	68	6485	8.67	1.01E–02
GO_0031625	Ubiquitin protein ligase binding	5	5.56	77	277	15,898	3.73	4.36E–02
Enrichment score: 1.59								
GO_0010803	Regulation of TNF-mediated signaling pathway	3	3.33	74	30	15,794	21.34	8.43E-03
R-HSA-5357956	TNFR1-induced NF-κB signaling pathway	3	3.33	49	30	8638	17.63	1.19E-02
R-HSA-5357905	Regulation of TNFR1 signaling	3	3.33	49	33	8638	16.03	1.43E–02
GO_0016874	Ligase activity	5	5.56	77	264	15,898	3.91	3.7E–02

ID, identifier; Pop, population.

[a]The 4 DAVID functional clusters are the (1) TNF/NF-κB signaling pathway, (2) NIK/NF-κB signaling pathway, (3) regulation of NF-κB signaling pathway, and (4) regulation of TNF-mediated signaling pathway. The criteria for the selected gene sets were (1) $P < .05$, (2) the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were not in the KEGG disease category, and (3) cluster enrichment score of >1.5.