Amphiregulin-producing T_H2 cells facilitate esophageal fibrosis of eosinophilic esophagitis

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Background: Massive eosinophil infiltration into the esophagus is associated with subepithelial fibrosis and esophageal stricture in patients with eosinophilic esophagitis (EoE). However, the pathogenesis of esophageal fibrosis remains unclear.
Objective: We sought to elucidate the cellular and molecular mechanisms underlying the induction of esophageal fibrosis.
Methods: We established a murine model of EoE accompanied by fibrotic responses following long-term intranasal administration of house dust mite antigen. Using this murine model, we investigated the characteristics of immune cells infiltrating the fibrotic region of the inflamed esophagus using flow cytometry and histological analyses. We also analyzed the local inflammatory sites in the esophagus of patients with EoE using single-cell RNA sequencing, flow cytometry, and immunohistochemistry.
Results: Enhanced infiltration of both amphiregulin-producing and IL-5–producing T_H2 cells was detected in the fibrotic area of the esophagus in mice subjected to repeated house dust mite exposure. Deletion of amphiregulin in $CD4^+$ T cells ameliorates esophageal fibrosis. An analysis of human esophageal biopsy samples showed that the infiltration of amphiregulin-producing $CD4^+$ T cells was higher in patients with EoE than in control patients. Furthermore, the number of infiltrated amphiregulin-producing $CD4^+$ T cells was associated with the degree of esophageal fibrosis in patients with EoE.
Conclusions: Amphiregulin, produced by T_H2 cells, contributes to esophageal fibrosis in EoE and may be a therapeutic target. (J Allergy Clin Immunol Global 2024;3:100287.)

Key words: *Eosinophilic esophagitis, $ST2^+$ T_H2 cells, amphiregulin, fibroblasts, esophageal fibrosis, single-cell RNA sequencing, IL-33, ST2*

Abbreviations used
EoE: Eosinophilic esophagitis
GERD: Gastroesophageal reflux disease
HDM: House dust mite
ILC2: Type 2 innate lymphoid cell
scRNA-seq: Single-cell RNA sequencing

Eosinophilic esophagitis (EoE) is a chronic inflammatory disease characterized by eosinophil-predominant inflammation, accompanied by tissue remodeling in the esophagus.[1-4] Esophageal remodeling includes subepithelial fibrosis, epithelial hyperplasia, angiogenesis, and smooth muscle hypertrophy, resulting in esophageal stenosis.[3,5] A stenotic esophagus causes dysphagia or food bolus impaction, which dramatically reduces the quality of life.[6] Furthermore, mechanical dilation with a balloon causes restenosis of the esophagus in more than half of the patients who underwent this treatment.[7,8] Fibrosis in the submucosal area of the esophagus, a hallmark of EoE, was found in 57% to 89% of pediatric patients and in 88% of adult patients.[9-12] Because esophageal fibrosis is irreversible in adult patients,[13] clarifying its mechanism is important for establishing novel treatments and prevention.

A long-term follow-up study of pediatric patients with EoE demonstrated that topical corticosteroid treatment improved esophageal fibrosis, suggesting that esophageal inflammation caused esophageal fibrosis.[14,15] Indeed, inflammatory cytokines produced by immune cells, such as eosinophils and T_H2 cells, play crucial roles in the development of esophageal fibrosis.[1,16-18] IL-5, which promotes the survival and activation of eosinophils, is also involved in esophageal fibrosis via eosinophils in mice.[17,19] Another T_H2 cytokine, IL-13, is highly expressed in the esophagus of patients with EoE and causes esophageal remodeling in IL-13 transgenic mice.[20-22] A subpopulation of T_H2 cells produces large amounts of IL-5 and IL-13 via IL-33 stimulation and exacerbates eosinophilic inflammation; thus, they are termed ST2 (IL-33 receptor)$^+$ T_H2 cells.[23,24] $ST2^+$ T_H2 cells also produce amphiregulin, an epidermal growth factor, and exacerbate tissue fibrosis by reprogramming eosinophils to produce osteopontin in inflamed lung.[25-27] Although amphiregulin contributes to the pathogenesis of tissue fibrosis in various organs,[28-33] its role and source in esophageal fibrosis remain unclear.

In the present study, we revealed the infiltration of amphiregulin-producing T_H2 cells in the fibrotic tissue of the esophagus in both patients with EoE and mice with esophageal inflammation. Deletion of amphiregulin in $CD4^+$ T cells ameliorated fibrotic responses in the inflamed esophagus. Furthermore, single-cell RNA sequencing (scRNA-seq) of the esophageal mucosa of patients with EoE showed enhanced infiltration of

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TABLE I. Study participants in Fig 1, *A-C*, and Fig 4, *G*

	Diagnosis	Age (y)	Sex	Allergy	Eos /HPF	$CD4^+$ cell /mm^2	Ratio of Sirius Red–stained area ($\mu m^2/\mu m^2$)	$Areg^+$ $CD4^+$ cell /mm^2
EoE group								
1	EoE	50	M	AD	107	357	0.76	81
2	EoE	46	F	AD/DA	36	146	0.86	202
3	EoE	53	M	None	30	94	0.65	210
4	EoE	51	M	None	55	173	0.73	—
5	EoE	57	M	AD	37	89	0.76	99
6	EoE	61	M	AR	40	271	0.73	25
7	EoE	50	M	AD	40	75	0.84	59
8	EoE	70	M	Asthma/AR/DA	35	92	0.59	178
9	EoE	53	F	None	246	192	0.85	272
10	EoE	20	M	None	150	452	0.80	395
11	EoE	62	M	DA	32	663	0.79	158
12	EoE	47	F	Asthma	37	194	0.85	183
13	EoE	40	M	Asthma/AD	113	556	0.88	200
14	EoE	60	M	AR	170	765	0.82	—
15	EoE	53	F	Asthma	58	457	0.68	153
16	EoE	24	M	Asthma/FA	30	320	0.72	—
17	EoE	47	M	Asthma	70	120	0.80	269
18	EoE	51	M	Asthma/FA	45	134	0.80	143
19	EoE	41	F	HES	343	163	0.92	—
Control group								
1	GERD/EMDs	78	M	None	0	51	0.74	15
2	GERD	65	M	None	0	45	0.49	10
3	GERD	72	M	None	0	25	0.56	59
4	GERD	72	M	None	0	42	0.62	37
5	GERD/EMDs	65	F	None	0	42	0.59	40
6	GERD/EMDs	55	M	None	0	65	0.65	7
7	GERD/EMDs	78	M	None	1	24	0.71	44

AD, Atopic dermatitis; *AR*, allergic rhinitis; *Areg*, amphiregulin; *DA*, drug allergy; *EMD*, esophageal motility disorder; *Eos*, eosinophil; *F*, female; *FA*, food allergy; *HES*, hypereosinophilic syndrome; *M*, male.

$ST2^+CD161^+CRTH2^+CD4^+$ cells with amphiregulin production, specifically in inflammatory lesions. These results indicate that amphiregulin-producing T_H2 cells are involved in shaping esophageal fibrosis in patients with EoE and may be novel therapeutic targets for the treatment or prevention of intractable complications in patients with EoE.

METHODS

Collection of esophageal biopsy samples and blood samples from patients

All participants with EoE fulfilled the international diagnostic criteria for EoE.[34] Multiple esophageal mucosal biopsies were collected for the diagnosis of EoE, regardless of the endoscopic findings. Two biopsies (4 in total) were collected from the distal esophagus (5 cm above the esophagogastric junction) and the midesophagus (15 cm above the esophagogastric junction). EoE was diagnosed when the eosinophilic infiltrate was greater than or equal to 15 eosinophils/HPF. None of the control participants diagnosed with esophageal motility disorders or gastroesophageal reflux disease (GERD) had EoE, asthma, and/or other allergic diseases. The study participants were men and women aged 20 to 78 years (Tables I and II). Esophageal fibrosis occurs primarily in the lamina propria or submucosa; these areas must be included in the biopsy samples for the analysis. Therefore, we selected 19 samples containing the lamina propria from 103 previously collected samples from patients with EoE (Table I). Similarly, we selected 7 of the 64 biopsy samples obtained from patients with control diseases (GERD or esophageal motility disorders without any allergic disorders) (Table I). Because esophageal fibrosis in patients with EoE did not change the rates of biopsies containing lamina propria,[35] we consider that the collection of samples in this study was not influenced by the tissue stiffness of the esophagus. When the biopsy samples were obtained, blood samples were also collected from the same patients.

The research proposals were reviewed by the Ethics Committee of Chiba University (registration no. 107). Informed consent was obtained from all the participants.

Data availability statement

The raw and analyzed data reported in this article are available under the accession number GEO: GSE249276.

For additional information, see this article's Online Repository at www.jaci-global.org.

RESULTS

Association of $CD4^+$ T-cell infiltration in the esophageal mucosa with esophageal fibrosis in human EoE and mouse allergic esophagitis models

To investigate whether esophageal tissue included fibrotic changes in patients with EoE, we analyzed esophageal mucosa biopsy samples. Sirius red staining revealed that fibrotic areas in the lamina propria of the esophageal mucosa in patients with EoE were significantly increased compared with those with GERD (Fig 1, *A*). The number of accumulated $CD4^+$ T cells was also

TABLE II. Study participants in Fig 4, *A-F*

	Diagnosis	Age (y)	Sex	Allergy	Eosinophil /HPF		Frequency of ST2$^+$ T_H2 cells (%)	Ratio of Sirius Red–stained area ($\mu m^2/\mu m^2$)
					At sample acquisition	(max)		
EoE group								
1	EoE	60	M	AR	170	(170)	4.18	0.82
2	EoE	54	F	Asthma	58	(58)	1.02	0.68
3	EoE	41	F	None	343	(343)	3.66	0.92
4	EoE	37	M	None	2	(30)	2.85	—
5	EoE	64	M	None	70	(140)	1.4	0.78
6*	EoE	24	M	Asthma	0	(30)	1.67	0.83
7	EoE	54	M	AD	2	(40)	1.93	0.88
8	EoE	51	F	Metal allergy	1	(188)	1.14	0.79
9	EoE	48	M	DA	0	(50)	0.31	—
10	EoE	73	M	Asthma/ECRS	100	(100)	2.48	0.82
11	EoE	68	M	None	8	(100)	1.04	0.77
12*	EoE	36	M	AR/AD	100	(100)	—	—
Control group								
1	EMDs	33	M	None	0	(0)	0.23	—
2	EMDs	72	M	None	0	(0)	0	—
3	EMDs	36	M	None	0	(0)	0.62	—
4	EMDs	29	M	None	0	(0)	0.49	—

AD, Atopic dermatitis; *AR*, allergic rhinitis; *DA*, drug allergy; *ECRS*, eosinophilic chronic rhinosinusitis; *EMD*, esophageal motility disorder; *F*, female; *M*, male.
*Nos. 6 and 12 in the EoE group were used for scRNA-seq analysis in Figs 4, *A-D*, and E4.

significantly increased in the lamina propria of the esophagus in patients with EoE compared with that in patients with GERD (Fig 1, *B*).

To investigate the cellular and molecular mechanisms underlying the induction of fibrotic responses in the esophagus, we used a murine model of esophageal fibrosis associated with EoE. Because recent reports have implicated that aeroallergens such as pollen or house dust mite (HDM) might trigger EoE,[36-38] we generated a model by long-term administration of the HDM antigen on the basis of previously published protocols[39] (see Fig E1, *A*, in this article's Online Repository at www.jaci-global.org). Histological analyses with hematoxylin and eosin staining revealed the characteristic phenotypes of EoE, including dilated intercellular spaces and basal zone hyperplasia[12,40] (Fig E1, *B*). Repeated exposure to HDM resulted in an increased number of eosinophils in the esophagus, where eosinophils infiltrated the intraepithelial region (Fig E1, *C-G*). Notably, the mice with repetitive HDM exposure showed enhanced fibrotic changes in the mucosal lamina propria and submucosa of the esophagus (Fig 1, *C*). The level of hydroxyproline, which reflects the amount of collagen, was also increased in mice subjected to repetitive exposure to HDM (Fig 1, *D*).

As detected in the patients with EoE, the number of infiltrated CD4$^+$ T cells was significantly increased in the fibrotic areas of the esophagus of the mice with repetitive exposure to HDMs (Fig 1, *E*). However, repetitive exposure to HDMs resulted in small differences in the numbers of B, CD8$^+$ T, and γδT cells in the esophagus (Fig E1, *H*). Thus, repetitive exposure to HDMs resulted in the induction of esophageal fibrotic responses accompanied by the characteristic histological changes in patients with EoE, such as dilated intercellular spaces, basal zone hyperplasia, and elevated numbers of intraepithelial eosinophils. Thus, infiltrated CD4$^+$ T cells in the esophagus are involved in esophageal fibrosis in both patients with EoE and mice with repetitive HDM exposure.

Accumulation of amphiregulin-producing T_H2 cells in the esophagus of mice with repetitive HDM exposure

A subpopulation of T_H2 cells, which show enhanced expression of ST2 and produce amphiregulin, contributes to shaping the pathology of fibrotic responses in the lung.[27] With regard to esophageal inflammation, mice with repetitive HDM exposure showed a significant increase in the infiltration of ST2$^+$ T_H2 cells in the esophagus (Fig 2, *A* and *B*), although the number of ST2$^+$ T_H2 cells was limited in the esophagus of the control mice (Fig 2, *A*, *left panel*). To identify amphiregulin-producing T_H2 cells *in vivo*, we generated amphiregulin reporter mice in which cells contained a tandem dimer red fluorescent protein (tdTomato) linked by the T2A sequence at the end of the amphiregulin gene (see Fig E2, *A*, in this article's Online Repository at www.jaci-global.org). The detection of the expression of amphiregulin by the reporter was comparable to detection by a specific antibody (Fig E2, *B*). In the esophagus of amphiregulin reporter mice with repetitive HDM exposure, ST2$^+$ T_H2 cells produced IL-5 or both IL-5 and amphiregulin, whereas ST2$^-$ T_H2 cells did not produce any amphiregulin or IL-5 (Fig 2, *C* and *D*). Some IL-5–producing T_H2 cells also produced IL-13 (Fig E2, *C*). Furthermore, histological analyses showed the accumulation of amphiregulin-producing CD4$^+$ T cells in the lamina propria, where many fibroblasts existed in the mice with repetitive HDM exposure (Fig 2, *E*, and Fig E2, *D*). Colocalization of amphiregulin-producing CD4$^+$ T cells and fibroblasts in the esophageal lamina propria indicates that amphiregulin may enhance the proliferation of esophageal fibroblasts in the esophagus. Indeed, amphiregulin stimulation *in vitro* resulted in

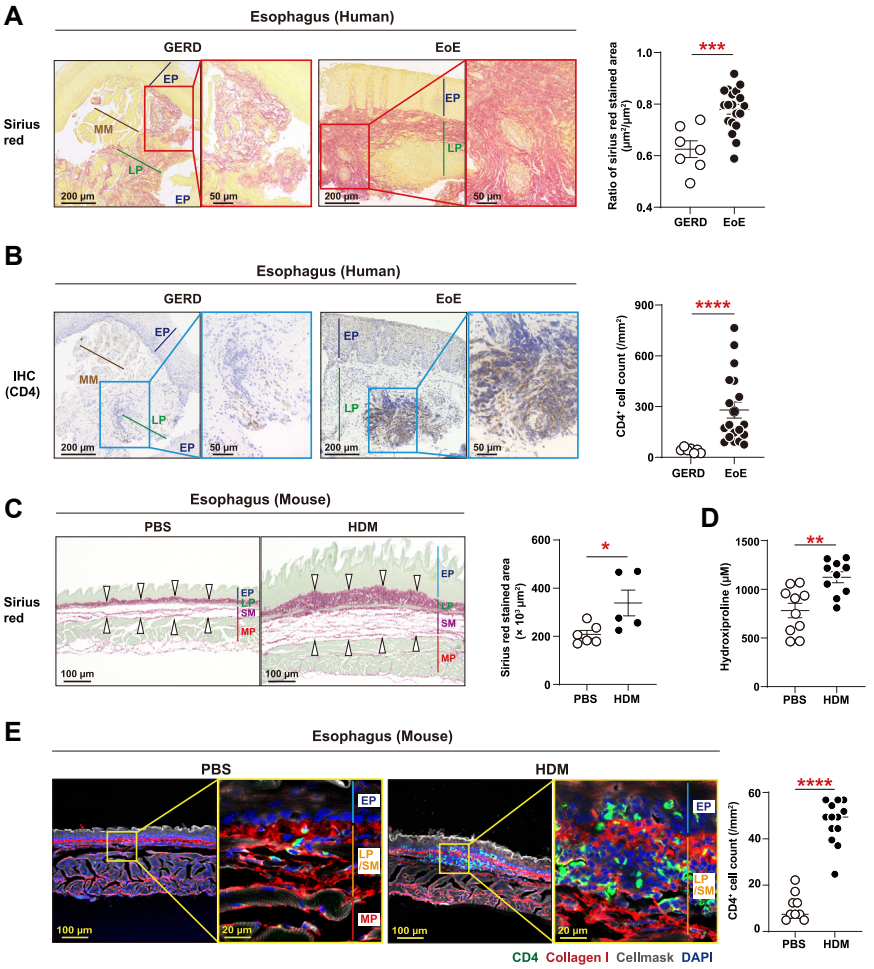

FIG 1. CD4$^+$ T cells associated with esophageal fibrosis in patients with EoE and mouse models. **A,** Representative images of the esophagus of patients with EoE or GERD. The samples were stained with Sirius red. The graph shows the ratio of fibrotic areas in randomly selected regions of the LP in the esophagus from patients with EoE (n = 19) or GERD/EMDs (n = 7) as controls. **B,** Representative images of the esophagus of patients with EoE or GERD. The brown colors indicate CD4$^+$ cells. The graph shows the count of CD4$^+$ cells in the images of the LP in the esophagus from patients with EoE (n = 19) or GERD/EMDs (n = 7). **C,** Representative images of the esophagus in mice treated with HDM extract (n = 5) or PBS (n = 6). The samples were stained with Sirius red. The graph shows fibrotic lesions in randomly selected areas stained with Sirius red. **D,** The concentration of hydroxyproline in the whole esophagus taken from mice treated with HDM (n = 10) or PBS (n = 10). **E,** Representative images of esophageal tissues stained with anti–collagen I antibody (red), anti-CD4 antibody (green), DAPI (blue), and cellmask (gray) from BALB/c mice treated with HDM (n = 13) or PBS (n = 9). The graph shows peak counts of CD4$^+$ cells/mm^2 in randomly selected regions. *C-E,* Three independent experiments were performed with similar results. *DAPI,* 4′-6-Diamidino-2-phenylindole, dihydrochloride; *EMD,* esophageal motility disorder; *EP,* epithelium; *IHC,* immunohistochemistry; *LP,* lamina propria; *MP,* muscularis propria; *SM,* submucosa. Comparisons of 2 groups were performed with Mann-Whitney *U* tests. Data are shown as the mean ± SEM. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001.

an increased number of Ki67-positive cells in the esophageal fibroblasts from naive mice (Fig 2, *F*). Thus, repetitive exposure to HDMs induced the infiltration of amphiregulin-producing T_H2 cells into the lamina propria, where amphiregulin enhanced the proliferation of esophageal fibroblasts.

Effects of CD4$^+$ T-cell–specific deficiency of amphiregulin on HDM-induced esophageal fibrotic responses during chronic allergic inflammation

We next determined whether or not amphiregulin contributed to esophageal fibrosis *in vivo*. To this end, we used amphiregulin-deficient (*Areg*$^{-/-}$) mice and repeatedly exposed them to HDM for 7 weeks (Fig 3, *A*). Genetic deletion of *Areg* resulted in ameliorated fibrotic responses accompanied by a decreased amount of hydroxyproline in the esophagus during HDM-induced chronic inflammation (Fig 3, *B* and *C*). However, the number of infiltrating eosinophils and CD4$^+$ T cells in the esophagus was comparable between *Areg*$^{+/+}$ and *Areg*$^{-/-}$ mice (see Fig E3, *A-C*, in this article's Online Repository at www.jaci-global.org).

To investigate whether amphiregulin produced by T_H2 cells is crucial for the induction of esophageal fibrosis, amphiregulin-sufficient or amphiregulin-deficient CD4$^+$ T cells were

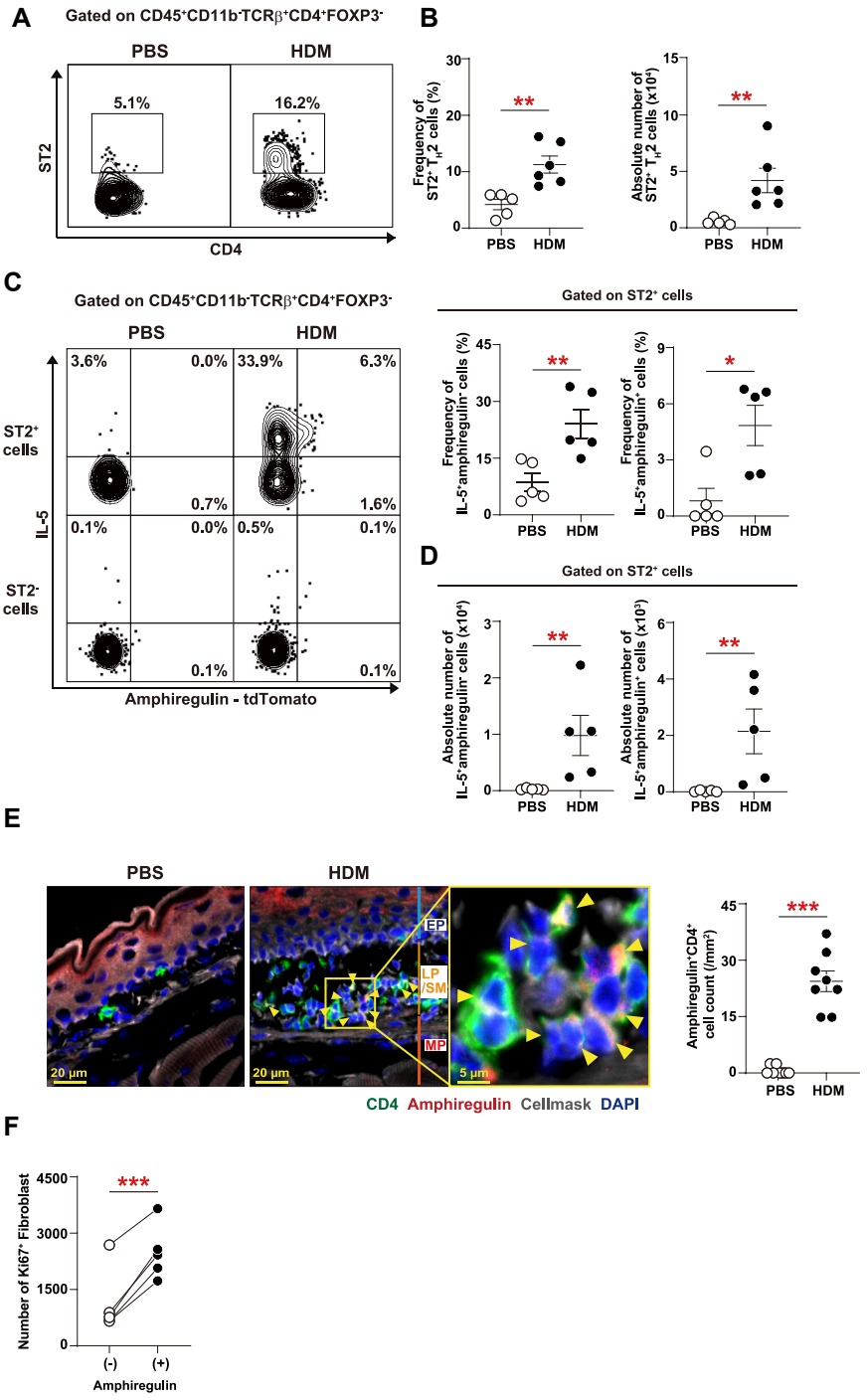

FIG 2. Amphiregulin-producing ST2$^+$ T_H2 cells were increased in the esophagus of the EoE mouse model. **A,** Representative flow cytometry plots of ST2$^+$ T_H2 cells (ST2$^+$CD4$^+$CD45$^+$CD11b$^-$TCRβ$^+$FOXP3$^-$) in the esophagus of BALB/c mice treated with HDM or PBS. The graphs on the right show the frequencies of ST2$^+$ T_H2 cells in the esophagus of the mice treated with HDM extract (n = 6) or PBS (n = 5). **B,** The graphs show the absolute numbers of ST2$^+$ T_H2 cells in the esophagus of the mice treated with HDM extract (n = 6) or PBS (n = 5). *A and B,* Three independent experiments were performed with similar results. **C,** Representative flow cytometry plots of cytokine profiles of ST2$^+$ or ST2$^-$ populations in CD45$^+$CD11b$^-$TCRβ$^+$CD4$^+$FOXP3$^-$ cells in the esophagus of amphiregulin reporter mice treated with HDM (n = 5) or PBS (n = 5). The graphs on the right show the frequencies of IL-5$^+$ amphiregulin$^-$ cells and IL-5$^+$ amphiregulin$^+$ cells among CD45$^+$CD11b$^-$TCRβ$^+$CD4$^+$FOXP3$^-$ST2$^+$ cells. **D,** The graphs show the absolute numbers of IL-5$^+$ amphiregulin$^-$ cells and IL-5$^+$ amphiregulin$^+$ cells among CD45$^+$CD11b$^-$TCRβ$^+$CD4$^+$FOXP3$^-$ST2$^+$ cells. **E,** Representative confocal micrographs of esophageal tissues stained with anti–red fluorescent protein antibody (red), anti–CD4 antibody (green), DAPI (blue), and cellmask (gray) from amphiregulin reporter mice treated with HDM (n = 8) or PBS (n = 8). Yellow arrowheads indicate amphiregulin and CD4 costained cells. The graph on the right shows counts of

adoptively transferred to *Areg*$^{-/-}$ mice exposed to HDM for 7 weeks (Fig 3, *D*). Adoptive transfer of amphiregulin-sufficient CD4$^+$ T cells resulted in the recovery of fibrotic responses in the esophagus of *Areg*$^{-/-}$ mice with repetitive exposure to HDM (Fig 3, *E* and *F*). The transfer of both *Areg*$^{+/+}$ and *Areg*$^{-/-}$ CD4$^+$ T cells enhanced the eosinophil infiltrations into the esophagus of *Areg*$^{-/-}$ mice following HDM exposure (Fig E3, *D*). Thus, amphiregulin produced by T$_H$2 cells is involved in the development of esophageal fibrosis in our experimental mouse model.

Amphiregulin-producing ST2$^+$ T$_H$2 cells in the esophageal mucosa of patients with EoE

Finally, we sought to determine whether amphiregulin-producing T$_H$2 cells were involved in the pathology of fibrotic responses in patients with EoE. To determine whether amphiregulin-producing T$_H$2 cells infiltrated the inflamed esophagus in patients with EoE, we performed scRNA-seq using samples of inflammatory or noninflammatory lesions of the esophagus from patients with EoE (Fig 4, *A*, patient #6 and #12 of the EoE group in Table II). Inflammatory and fibrotic lesions showed white plaques and furrows, whereas noninflammatory lesions showed no typical findings (Fig 4, *A*).

To characterize immune cells in the esophageal biopsies from patients with EoE, scRNA-seq profiles were generated using 5528 CD45$^+$ immune cells collected from the noninflamed and inflamed areas of the esophagus of 2 patients. Overall, we identified 8 clusters in an unbiased manner based on differentially expressed genes, and inferred cluster identities based on the expression of marker genes (Fig 4, *B*; see Fig E4, *A*, in this article's Online Repository at www.jaci-global.org). We did not detect a population of type 2 innate lymphoid cells (ILC2s; designated as *IL7R*$^+$ *CD3G*$^-$) in our scRNA-seq data. Four immune populations, including CD4$^+$ T cells, were detected in higher frequencies in inflammatory lesions than in noninflammatory lesions in the esophagus of patients with EoE (Fig 4, *C*). To investigate the CD4$^+$ T-cell population, we extracted and reclustered the CD4$^+$ T-cell clusters (Fig 4, *D*). Among the infiltrating CD4$^+$ T cells in the esophagus, a subpopulation with a high expression of *IL1RL1* (which encodes ST2), *PTGDR2* (CRTH2), *KLRB1* (CD161), *GATA3*, *CD4*, and *CD3G*,[41] but not *FOXP3*, showed the enhanced expression of *AREG*, which encodes amphiregulin (Fig 4, *D*). This subpopulation was observed in both patients with EoE that were analyzed (Fig E4, *B*). To further substantiate these findings, we reanalyzed a publicly available scRNA-seq data set from a study by Morgan et al[42] related to EoE (GSE175930). Through this reanalysis, we identified 662 CD4$^+$ T cells and confirmed the presence of a ST2$^+$ T$_H$2-cell subset, similarly characterized by the high expression of *AREG*. These concordant results provided consistent evidence for the existence of an amphiregulin-high–producing ST2$^+$ T$_H$2-cell population in EoE (Fig E4, *C*).

Fluorescence-activated cell sorting analyses showed the infiltration of CD3$^+$CD4$^+$CD45RO$^+$CD27$^-$CRTH2$^+$CD161$^+$ cells[43] in the mucosal tissues of patients with EoE but not in PBMCs, whereas we detected a small number of CD3$^+$CD4$^+$CD45RO$^+$ CD27$^-$CRTH2$^+$CD161$^+$ cells in both mucosal tissues and PBMCs from control groups (Fig 4, *E*, and Table II). Importantly, the frequencies of CD3$^+$CD4$^+$CD45RO$^+$CD27$^-$CRTH2$^+$ CD161$^+$ cells in the esophageal mucosa correlated with the area of fibrotic tissues in the biopsy samples (Fig 4, *F*). An immunohistological analysis demonstrated that amphiregulin-producing CD4$^+$ T cells infiltrated the epithelium and mucosal area in the esophageal tissues of patients with EoE, whereas few of these cells were observed in patients with GERD (patient #1-3, 5-13, 15, 17, and 18 of the EoE group and patient #1-7 of the GERD group in Table I) (Fig 4, *G*). The number of infiltrating cells in the esophagus was significantly higher than in patients with GERD (Fig 4, *G*). The number of amphiregulin-producing CD4$^+$ T cells was reduced in the remission phase (patient no. #2, 5, 11, 13, and 17 of the EoE group in Tables I and III) (Fig 4, *H*). These results indicated that amphiregulin-producing T$_H$2 cells likely play a pathological role in esophageal fibrosis in patients with EoE.

DISCUSSION

In the present study, we found that a unique subpopulation of T$_H$2 cells with specific amphiregulin production infiltrated the mucosa of the esophagus and was involved in shaping fibrotic responses during eosinophilic inflammation in mice and humans. In a mouse model of EoE, CD4$^+$ T-cell–specific amphiregulin deficiency ameliorated fibrotic responses in the inflamed esophagus. Amphiregulin-producing CD3$^+$CD4$^+$CD45RO$^+$CD27$^-$ CRTH2$^+$CD161$^+$ cells were detected in the esophageal tissues of patients with EoE and were associated with the degree of esophageal fibrotic responses.

Amphiregulin-producing CD4$^+$ T cells infiltrated the lamina propria or submucosal layer where esophageal fibrosis occurred (Fig 2, *E*). Amphiregulin induced the proliferation of fibroblasts (Fig 2, *F*) and is required for collagen and α-smooth muscle actin expression by TGF-β stimulation.[28,32,33,44] Thus, amphiregulin produced by ST2$^+$ T$_H$2 cells may directly increase the number of fibroblasts in the esophageal mucosa, resulting in an increase in the amount of fibrotic molecules. In lung and intestinal fibrosis models, the regulation of fibroblasts by amphiregulin produced by eosinophils, mast cells, dendritic cells, or T$_H$17 cells was reported.[28,32,44,45] Thus, the association between fibroblasts and immune cells via amphiregulin is essential for the development of fibrotic diseases.

An effective treatment for reducing esophageal inflammation in patients with EoE is the administration of topical steroids.[46] EoE is often refractory to steroid treatment and frequently to recur.[47,48] ST2$^+$ T$_H$2 cells are steroid resistant in IL-33–induced eosinophilic inflammation.[49] Thus, the ST2$^+$ T$_H$2 cells remaining in the inflamed esophagus after steroid treatment may cause recurrent eosinophilic inflammation. Furthermore, amphiregulin

amphiregulin-producing CD4$^+$ cells in randomly selected regions of the LP and SM. **F,** The graph shows the absolute number of Ki67-positive fibroblasts with or without amphiregulin stimulation *in vitro*. Fibroblasts isolated from 5 mice were cultured for 24 hours. The 2 dots connected by a line indicate the cells isolated from the same mice. *C-F,* Two independent experiments were performed with the same results. *DAPI*, 4′-6-Diamidino-2-phenylindole, dihydrochloride; *EP*, epithelium; *LP*, lamina propria; *MP*, muscularis propria; *SM*, submucosa. Comparisons of 2 groups were calculated with paired *t* tests or Mann-Whitney *U* tests. Data are shown as the mean ± SEM. **P* < .05, ***P* < .01, ****P* < .001.

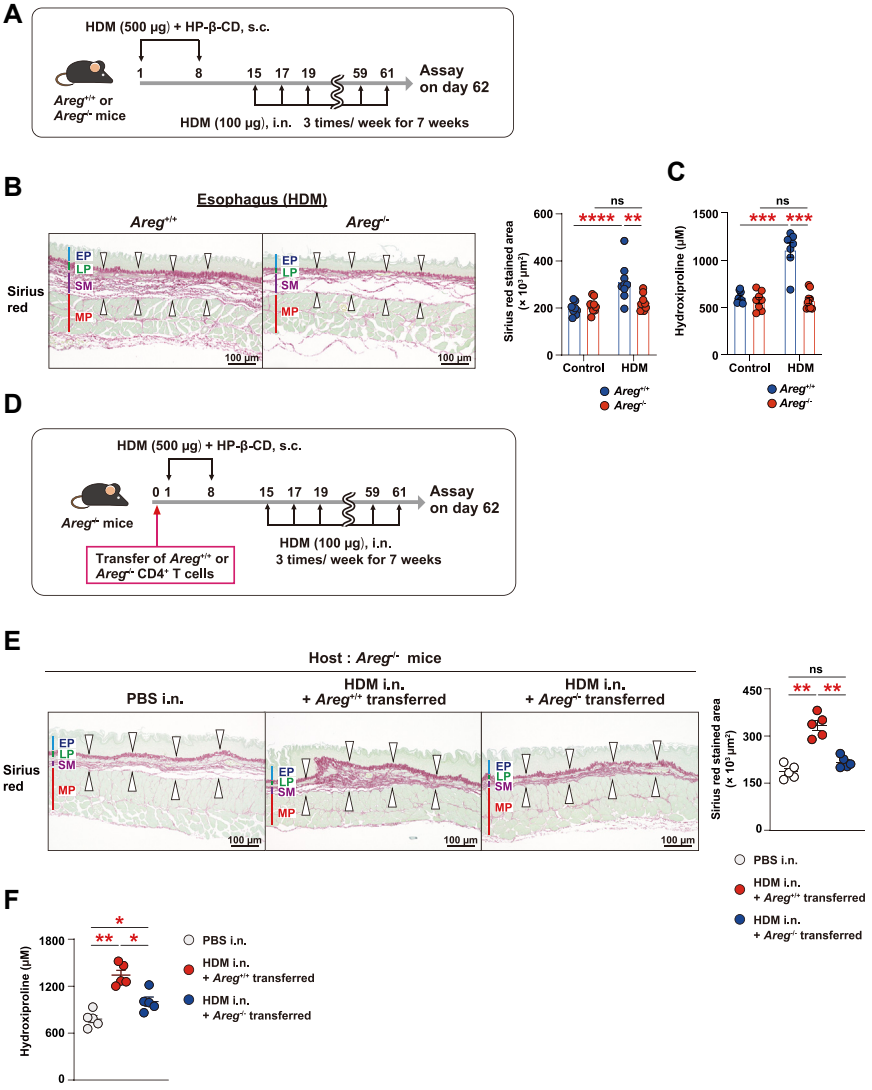

FIG 3. Amphiregulin production from $CD4^+$ T cells is critical for the development of esophageal fibrosis in EoE. **A,** Experimental protocol for the induction of esophageal fibrosis accompanied by EoE in $Areg^{+/+}$ or $Areg^{-/-}$ mice by long-term exposure to HDM. **B,** Representative images of the esophagus in $Areg^{+/+}$ mice and $Areg^{-/-}$ mice with HDM exposure. The esophageal samples were stained with Sirius red. The graph shows fibrotic areas in randomly selected regions stained with Sirius red (n = 9/group). **C,** Hydroxyproline concentration in the esophagus of $Areg^{+/+}$ mice treated with HDM (n = 7) or PBS (n = 8) and $Areg^{-/-}$ mice treated with HDM (n = 9) or PBS (n = 8). **D,** Experimental protocol to examine whether or not amphiregulin produced from $CD4^+$ T cells is critical for the development of esophageal fibrosis. Ten million $CD4^+$ T cells collected from the spleens of $Areg^{+/+}$ or $Areg^{-/-}$ mice were transferred into $Areg^{-/-}$ mice before HDM treatment. **E,** Representative images of the esophagus in $Areg^{-/-}$ mice treated with HDM following $Areg^{+/+}$ or $Areg^{-/-}$ $CD4^+$ T-cell transfer and in nonsensitized $Areg^{-/-}$ mice without $CD4^+$ T-cell transfer. The graph shows the ratios of fibrotic areas randomly selected in the LP and SM (n = 5/group). The esophageal samples were stained with Sirius red. **F,** Hydroxyproline concentration in the esophagus of $Areg^{-/-}$ mice transferred with $Areg^{+/+}$ or $Areg^{-/-}$ $CD4^+$ T cells and treated with HDM and control mice (n = 5/group). *A-F,* Two independent experiments were performed with similar results. *EP,* Epithelium; *LP,* lamina propria; *MP,* muscularis propria; *ns,* not significant; *SM,* submucosa. Each symbol represents an individual mouse. Comparisons of 2 groups were calculated with Mann-Whitney *U* tests. Data are shown as the mean ± SEM. $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$.

production from the remaining $ST2^+$ T_H2 cells may exacerbate fibrosis in the esophagus. Indeed, several patients in this study showed an elevated number of $CD3^+CD4^+CD45RO^+CD27^-CRTH2^+CD161^+$ cells and tissue fibrosis despite low eosinophil counts in the esophageal biopsy tissue (patients #4, 6, 7, 8, 9, and 11 of the EoE group in Table II). This may explain why steroid administration does not inhibit the progression of esophageal fibrosis, regardless of the reduction in eosinophils in the esophagus.[13,50]

IL-33 stimulation of $ST2^+$ T_H2 cells induces the production of various inflammatory molecules including IL-5, amphiregulin, and calcitonin gene–related peptide.[27,51,52] In the esophagus,

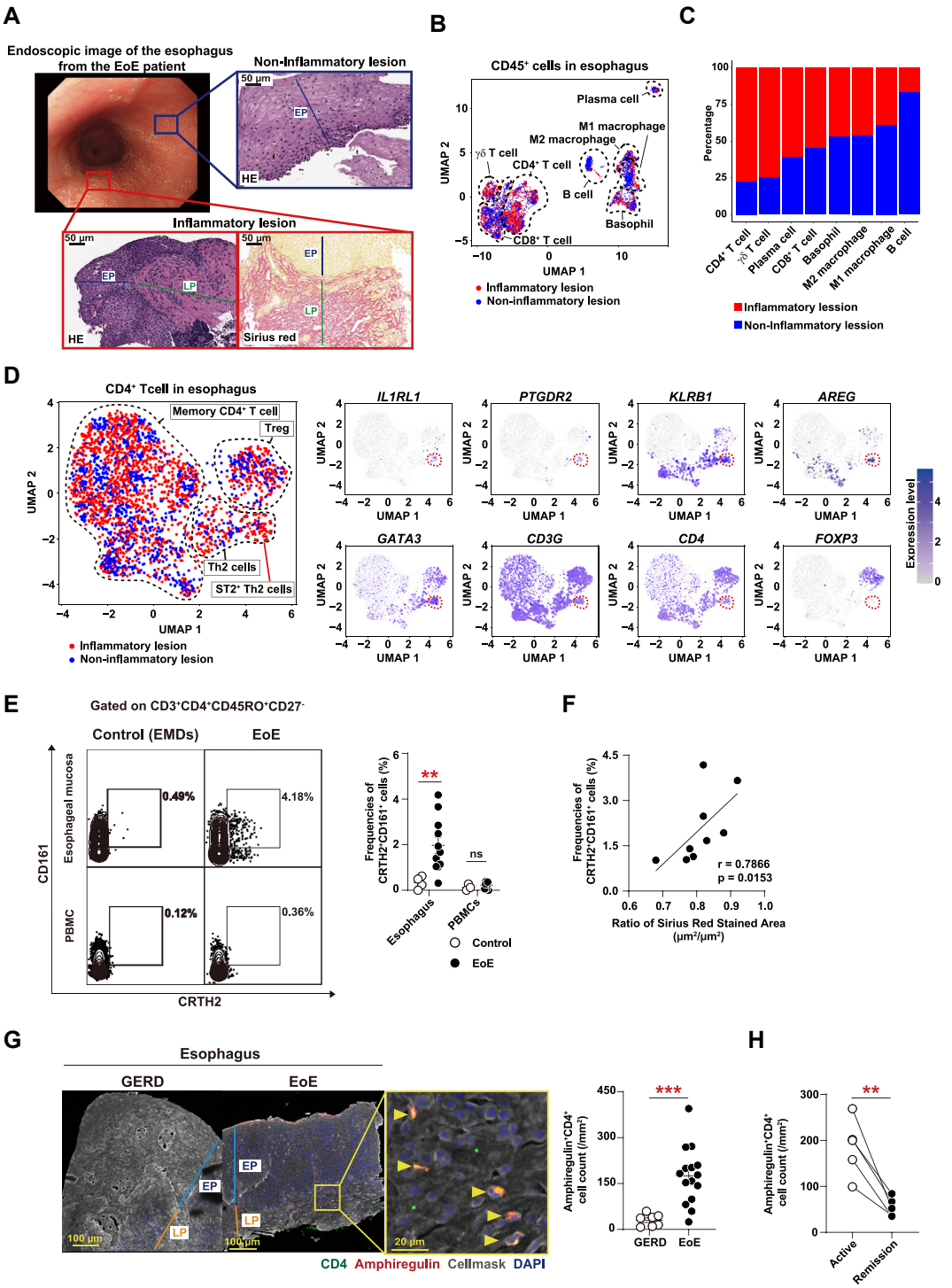

FIG 4. Amphiregulin-producing ST2$^+$ T_H2 cells in the esophageal mucosa of patients with EoE. **A,** Endoscopic and histological images of the esophagus in patient no. 6 of the EoE group in Table II, whose esophageal mucosal biopsy samples were analyzed with scRNA-seq. The red square indicates an inflammatory lesion, and the blue square indicates a noninflammatory lesion. A histological analysis was performed by HE or Sirius red staining. **B,** A UMAP of cells from the esophagus biopsy samples of the patients with EoE. CD45$^+$ cells are depicted and framed according to cellular subset. Blue symbols indicate the cells in the noninflammatory lesion, and red symbols indicate the cells in the inflammatory lesion. **C,** The percentages of each cellular subset in the biopsy samples. Blue bars indicate cells in noninflammatory lesions. Red bars indicate cells in inflammatory lesions. **D,** (*Left panel*) UMAP of CD4$^+$ T cells in noninflammatory lesions (blue) and inflammatory lesions (red). The indicated gene expression in CD4$^+$ T cells is shown (*right panels*). The clusters enclosed by the red lines represent ST2$^+$ T_H2 cells. **E,** Representative flow cytometry plots of CD161$^+$ and CRTH2$^+$ cells gated on CD3$^+$CD4$^+$CD45RO$^+$CD27$^-$ cells in human esophageal mucosal tissues and PBMCs from patients with EMDs or EoE. The graph shows the frequency of ST2$^+$ T_H2 cells in

TABLE III. Study participants in Fig 4, *H*

Case nos. in Table I	Treatment	Active		Remission	
		Eos/HPF	Areg$^+$ CD4$^+$ cell /mm^2	Eos/HPF	Areg$^+$ CD4$^+$ cell /mm^2
2	PPI	36	202	1	67
5	PPI	37	99	1	37
11	PPI	32	158	8	35
13	Benralizumab*	113	200	4	84
17	PPI	70	269	3	52

Areg, Amphiregulin; *Eos*, eosinophil; *PPI*, proton pump inhibitor.
*Benralizumab: an anti–IL-5 receptor α mAb. No. 13 participated in the MESSINA trial (a multicenter, randomized, double-blind, parallel-group, placebo controlled study to investigate the use of benralizumab for eosinophilic esophagitis).

the IL-33 expression is higher in the esophageal epithelium of patients with EoE than in healthy individuals.[53,54] Furthermore, exogenous treatment of wild-type mice with IL-33 increased amphiregulin expression in the esophagus.[53] Thus, ST2$^+$ T$_H$2 cells infiltrating the lamina propria or submucosa may be exposed to IL-33, leading to the excessive production of amphiregulin in the esophagus. Therefore, instead of steroid treatment, neutralization of IL-33 or inhibition of its binding to its receptor may provide a new therapeutic approach to esophageal fibrosis.

Besides CD4$^+$ T cells, eosinophils, mast cells, or ILC2s are the most likely amphiregulin-producing cells involved in the pathogenesis of esophageal fibrosis in EoE.[26,45,55,56] Owing to technical difficulties, eosinophils and mast cells were not detected by our scRNA-seq analysis. Flow cytometry showed that ILC2s (Lineage$^-$ CRTH2$^+$) increased with the number of infiltrated eosinophils in the esophageal epithelium in patients with EoE.[57] We collected the samples from an inflamed area of the esophagus in the 2 patients with active EoE using an endoscope (Fig 4, *A*). Despite samples being collected from the inflammatory site, ILC2s, classified by the expression of *IL7R* but not *CD3G*, were not detected by scRNA-seq. Similarly, scRNA-seq analysis of esophageal samples by other groups did not detect ILC2s.[42,55,58] In particular, the study by Morgan et al mentioned the rarity of ILC2s and the small size of the biopsy samples as reasons why ILC2s could not be detected by scRNA-seq.[42] Further technical advances are required to detect minor populations of small cell numbers for the analysis of esophageal samples.

Our findings provide new insights into the roles of amphiregulin and ST2$^+$ T$_H$2 cells in the pathogenesis of esophageal fibrosis accompanied by EoE. The presence of amphiregulin and/or ST2$^+$ T$_H$2 cells in the esophagus may be an indicator of esophageal fibrosis, and therapies targeting these cells may ameliorate esophageal fibrosis.

DISCLOSURE STATEMENT

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Clinical implications: The evaluation of amphiregulin-producing CD4$^+$ T cells in esophageal biopsy samples is useful for diagnosing esophageal fibrosis, and these cells may be a novel target for therapy.

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esophageal mucosal tissues or PBMCs from patients with EMDs (n = 4) or EoE (n = 11). **F,** Correlation diagram between the ratio of fibrotic areas stained with Sirius red and the frequency of ST2$^+$ T$_H$2 cells in esophageal mucosal tissues. **G,** Representative confocal micrographs of esophageal tissues stained with antiamphiregulin antibody (red), anti-CD4 antibody (green), DAPI (blue), and cell mask (gray) from patients with EoE (n = 15) or patients with GERD (n = 7). Yellow arrowheads indicate amphiregulin and CD4 costained cells. The right graph shows the counts of amphiregulin-producing CD4$^+$ cells in randomly selected regions of the epithelium, lamina propria, and muscularis mucosa. Each symbol represents an individual patient. **H,** The counts of amphiregulin-producing CD4$^+$ cells in the esophagus from the same patients (n = 5) in the active and remission phases. The areas were randomly selected regions of the epithelium, lamina propria, and muscularis mucosa. Dots connected by lines indicate the same patients. Comparisons of 2 groups were calculated with paired *t* tests and Mann-Whitney *U* tests. The correlation coefficient was calculated with Spearman rank correlation coefficient. *DAPI*, 4′-6-Diamidino-2-phenylindole, dihydrochloride; *EMD*, esophageal motility disorder; *EP*, epithelium; *HE*, hematoxylin and eosin; *LP*, lamina propria; *ns*, not significant; *UMAP*, uniform manifold approximation and projection. Data are shown as the mean ± SEM. $^{**}P < .01$, $^{***}P < .001$.

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