

Active eosinophilic esophagitis is characterized by epithelial barrier defects and eosinophil extracellular trap formation

D. Simon^{1,*}, S. Radonjic-Hösli^{2,*}, A. Straumann³, S. Yousefi² & H.-U. Simon²

¹Department of Dermatology, Inselspital, Bern University Hospital; ²Institute of Pharmacology, University of Bern, Bern; ³Swiss EoE Research Network, Olten, Switzerland

To cite this article: Simon D, Radonjic-Hösli S, Straumann A, Yousefi S, Simon H-U. Active eosinophilic esophagitis is characterized by epithelial barrier defects and eosinophil extracellular trap formation. *Allergy* 2015; **70**: 443–452.

Keywords

antimicrobial peptides; eosinophil extracellular traps; eosinophilic esophagitis; eosinophils; epithelial barrier.

Correspondence

Dagmar Simon, MD, Department of Dermatology, Inselspital, University of Bern, CH-3010 Bern, Switzerland.

Tel.: +41-31-632-2278

Fax: +41-31-632-2233

E-mail: dagmar.simon@insel.ch

*These authors contributed equally to this study.

Accepted for publication 25 December 2014

DOI:10.1111/all.12570

Edited by: Thomas Bieber

Abstract

Background: Eosinophilic esophagitis (EoE) exhibits esophageal dysfunction owing to an eosinophil-predominant inflammation. Activated eosinophils generate eosinophil extracellular traps (EETs) able to kill bacteria. There is evidence of an impaired barrier function in EoE that might allow pathogens to invade the esophagus. This study aimed to investigate the presence and distribution of EETs in esophageal tissues from EoE patients and their association with possible epithelial barrier defects.

Methods: Anonymized tissue samples from 18 patients with active EoE were analyzed. The presence of DNA nets associated with eosinophil granule proteins forming EETs and the expression of filaggrin, the protease inhibitor lympho-epithelial Kazal-type-related inhibitor (LEKTI), antimicrobial peptides, and cytokines were evaluated by confocal microscopy following immune fluorescence staining techniques.

Results: Eosinophil extracellular trap formation occurred frequently and was detected in all EoE samples correlating with the numbers of infiltrating eosinophils. While the expression of both filaggrin and LEKTI was reduced, epithelial antimicrobial peptides (human beta-defensin-2, human beta-defensin-3, cathelicidin LL-37, psoriasin) and cytokines (TSLP, IL-25, IL-32, IL-33) were elevated in EoE as compared to normal esophageal tissues. There was a significant correlation between EET formation and TSLP expression ($P = 0.02$) as well as psoriasin expression ($P = 0.016$). On the other hand, a significant negative correlation was found between EET formation and LEKTI expression ($P = 0.016$).

Conclusion: Active EoE exhibits the presence of EETs. Indications of epithelial barrier defects in association with epithelial cytokines are also present which may have contributed to the activation of eosinophils. The formation of EETs could serve as a firewall against the invasion of pathogens.

Abbreviations

AD, Atopic dermatitis; C5a, Complement factor 5a; EET, Eosinophil extracellular trap; EoE, Eosinophilic esophagitis; EPX, Eosinophil peroxidase; HBD, Human beta-defensin; IL, Interleukin; LEKTI, Lympho-epithelial Kazal-type-related inhibitor; LPS, Lipopolysaccharides; NE, Normal esophagus; PAMPs, Pathogen-associated molecular patterns; PAR, Proteinase-activated receptor; PI, Propidium iodide; Th, T-helper; TLR, Toll-like receptor; TSLP, Thymic stromal lymphopoietin.

Eosinophilic esophagitis (EoE) is a chronic inflammatory disease characterized by symptoms of esophageal dysfunction and associated with an eosinophil-predominant infiltration of inflammatory cells in the esophageal mucosa (1). Among EoE patients, 75% have concomitant allergic diseases of the airways and/or skin, and up to 90% have IgE sensitization to food and aeroallergens (2, 3). As with other atopic diseases, in addition to eosinophils, the inflammation typically exhibits an infiltration of T cells and mast cells as well as a

T-helper (Th) 2 cytokine expression pattern (4). Although allergens have been identified as potential triggers of the disease, the factors that perpetuate the inflammation in EoE are not completely understood.

Activated eosinophils have been shown to generate eosinophil extracellular traps (EETs) capable of killing bacteria in the extracellular space (5, 6). Such DNA nets occur in association with eosinophil granule proteins and are formed after priming with interleukin (IL)-5, interferon (IFN)- γ or adhesion molecules followed by activation with complement factor 5a (C5a), lipopolysaccharides (LPS), *Staphylococcus aureus*, or thymic stromal lymphopoietin (TSLP) (5–7). EETs have been identified in various infectious, but also noninfectious, diseases, including allergic diseases, such as bronchial asthma and atopic dermatitis (AD) (5, 8, 9).

Epithelial barrier defects have long been recognized as a key factor in the development of bronchial asthma and AD (10, 11). Recently, a reduced expression of tight junction proteins and desmoglein (DSG)-1, resulting in a loss of epithelial integrity, has been reported also for active EoE (12, 13). Subsequent to impaired barrier function, environmental factors, including microbes and their products, allergens, or irritants, can induce immune responses by directly activating epithelial cells, for example, via pattern-recognition receptors, to produce and release antibacterial peptides and cytokines (14, 15). Epithelial cytokines, such as TSLP, IL-25, and IL-33, can stimulate innate immune cells, such as eosinophils, basophils, and dendritic cells (6, 16, 17). In addition, TSLP, IL-25, and IL-33 are known to skew Th2 immune responses, resulting in eosinophil infiltration and activation (16, 18–20).

Th2 cytokines have been shown to additionally impair the epithelial barrier in AD as they caused reduced filaggrin production by keratinocytes (21). Moreover, whereas IL-17 enhanced the expression of human antimicrobial peptides by keratinocytes, Th2 cytokines, such as IL-4 and IL-13, diminished their production (22–24).

We hypothesized that in active EoE, eosinophils are activated, at least partially, as a consequence of epithelial barrier defects and the subsequent innate immune responses, resulting in EET formation. Therefore, we investigated the presence and distribution of EETs in EoE esophageal tissues, asking whether EET formation was associated with the clinical and endoscopic features of EoE. To study whether such EET formation also correlated with epithelial barrier dysfunction, we additionally analyzed the expression of filaggrin, the protease inhibitor lympho-epithelial Kazal-type-related inhibitor (LEKTI), antimicrobial peptides, and cytokines in the esophagus of patients suffering from active EoE.

Methods

Esophageal specimens

Anonymized biopsy tissues of 18 patients archived as paraffin-embedded samples from a previous clinical trial (registered at www.ClinicalTrials.gov NCT01056783) were analyzed (25). All patients had active EoE confirmed clinically, endoscopically, and histologically, without current

therapy for EoE (Table S1 in Supporting Information). Normal tissue was obtained from three patients without esophageal disease who were undergoing diagnostic endoscopy. Written informed consent was obtained from all patients prior to enrollment in the study. The study was approved by the local ethics committee and the regulatory authority in Switzerland, SwissMedic. Clinical symptoms and endoscopic findings were assessed and scored by the gastroenterologist (A.S.). Because of the patchy nature of EoE, eight individual biopsies from the proximal and distal esophagus (each $n = 4$) were analyzed for each patient.

Fluorescence staining and confocal microscopy

Staining was performed on 6- μ m sections of 4% formaldehyde-fixed and paraffin-embedded esophageal biopsy tissue. Cellular infiltration and cytokine expression were analyzed by immunofluorescence techniques as described previously (26) using antibodies directed against eosinophil peroxidase (EPX; Lee Laboratory, Mayo Clinic, AZ, USA), filaggrin, human beta-defensin (HBD)-2, psoriasin (protein S100A7) (all Abcam, Cambridge, UK), LEKTI, LL-37, HBD-3, IL-25, IL-32 (all from Novus Biologicals, Littleton, CO, USA), IL-23 (BioLegend, San Diego, CA, USA), IL-33 (Enzo Life Science, Lausen, Switzerland), IL-17, TSLP (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD3, and mast cell tryptase (both from Dako, Glostrup, Denmark). Appropriate secondary antibodies labeled with Alexa Fluor 488 (Invitrogen Molecular Probes, Paisley, UK) or Cy3 (Jackson ImmunoResearch Ltd, Suffolk, UK) were applied. DNA was visualized with propidium iodide (PI; Molecular probes, Eugene, OR, USA). Evaluation was performed using a confocal laser scanning microscope (LSM 510; Carl Zeiss Jena GmbH, Jena, Germany).

Eosinophils and EETs were counted in five fields with high inflammatory activity per biopsy corresponding to a total of 40 fields (0.34 mm²) per patient at a magnification $\times 1000$. Clustered and disseminated eosinophils were analyzed separately. Psoriasin, IL-25-, IL-32-, and IL-33-expressing cells were evaluated similarly.

Epithelial barrier protein and cytokine expression values were obtained by scoring the intensity and distribution of the staining (0 = none; 1 = mild; 2 = medium; 3 = strong, 4 = very strong) in areas with high inflammatory activity. To quantify the intensity of filaggrin expression, the area in pixel software Image-Pro Plus (Media Cybernetics, Rockville, MD, USA) was used.

Statistics

Mean values ($\pm$ SEM) are provided. For comparisons of EoE vs normal esophagus (NE) or between EoE subgroups, the unpaired *t*-test was applied. To describe the correlation between two variables, Pearson's coefficient (*r*) and, for ordinal data, ANOVA were determined. *P* values < 0.05 were considered statistically significant. Statistical analysis was performed using GRAPH PAD software PRISM 6 (La Jolla, CA, USA).

Results

Eosinophil extracellular traps are formed in active EoE

As EETs have been observed in eosinophilic skin and airway diseases (8, 9), we searched for eosinophils generating extracellular DNA traps in EoE. Indeed, we detected EET-positive eosinophils in both the proximal and distal esophagus of EoE patients (Fig. 1A). Approximately 15% (range 2–33%) of the eosinophils infiltrating the esophageal epithelium were found to generate EETs. The confocal microscopy images clearly showed the extracellular DNA nets in association with the granular protein EPX (Fig. 1A). The numbers of EET-positive eosinophils were highest in the central part of the epithelium, as compared with those in the apical (luminal) and basal parts ($P < 0.0001$). In the central and basal parts of the epithelium, we mostly observed disseminated and solitary eosinophils, whereas in the apical part, eosinophils were often seen in clusters (see Fig. S1 in Supporting Information). We also noticed that EETs were usually generated by disseminated eosinophils and to a lesser extent by the clustered eosinophils ($P < 0.0001$). The number of EET-positive eosinophils correlated with the total number of infiltrating eosinophils ($r = 0.3$; $P = 0.008$) (Fig. 1B).

As with the eosinophilic skin diseases, in which eosinophil granular deposits in addition to EETs have been observed (8), we found substantial eosinophil degranulation in EoE.

The numbers of EET-positive eosinophils also correlated in a significant manner with the extent of granule deposits ($r = 0.26$; $P = 0.0001$) (Fig. 1C).

Evidence for epithelial barrier defects in active EoE

An impaired epithelial barrier function in particular mutations of filaggrin and/or its reduced production have been linked to AD and asthma (27, 28). We observed in NE a bandlike granular filaggrin expression in the uppermost cell layers of the epithelium, although the overall expression of filaggrin in the esophagus was much diminished as compared to normal skin (Fig. 2A). In EoE, the expression of filaggrin was reduced compared with NE (proximal, $P = 0.0025$; distal, $P = 0.006$) and could be detected in single cells only (Fig. 2A).

A reduced enzymatic activity of LEKTI, a protease inhibitor responsible for the homeostasis of the epithelium, has been associated with barrier defects in Netherton syndrome and allergic diseases (29, 30). In NE, LEKTI was continuously expressed by all cells in the apical half of the epithelium, whereas in EoE, the expression was significantly reduced toward a bandlike (medium, score 2) or interrupted (mild, score 1) superficial expression (proximal esophagus, $P = 0.005$; distal esophagus, $P = 0.004$) (Fig. 2B). Low expression of LEKTI was associated with high numbers of

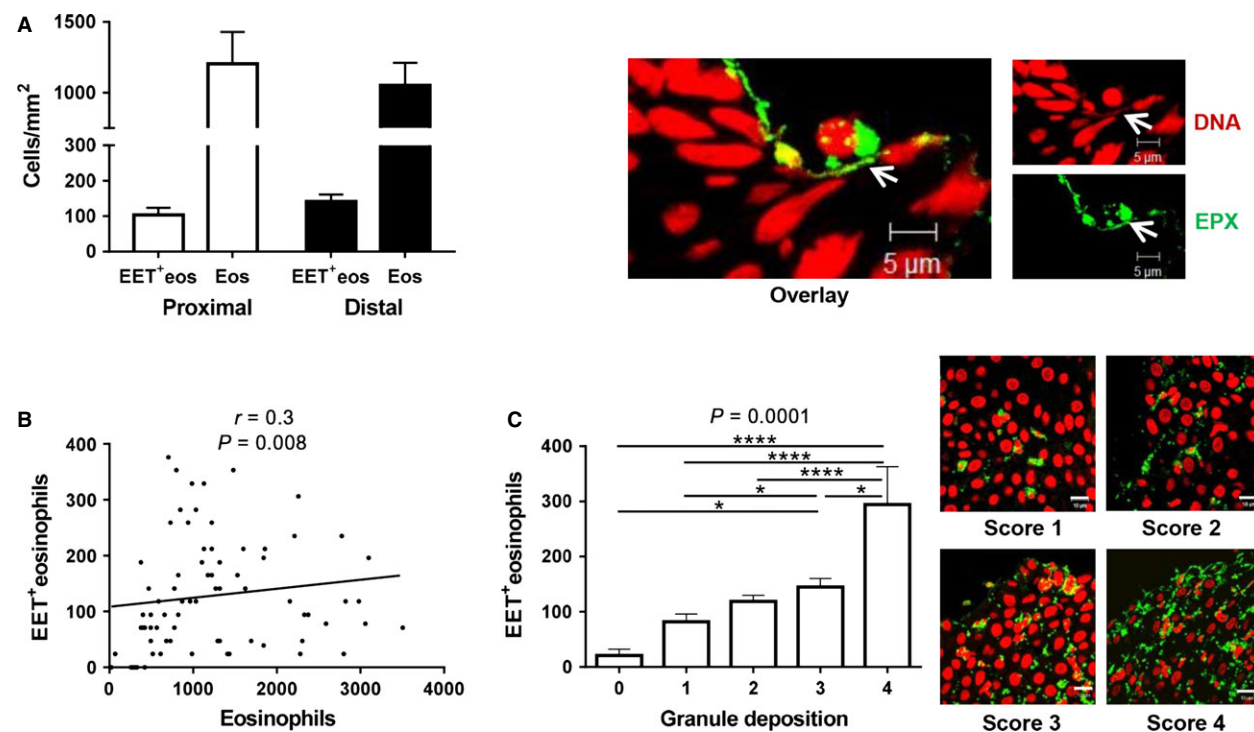


Figure 1 EETs are found in EoE specimens. (A) The number of eosinophils infiltrating the esophageal epithelium and the fraction thereof forming EETs were determined ($n = 10$). Right: Representative image of an EET (arrows). Eosinophils were identified using

anti-EPX mAb. DNA was stained with propidium iodide. Bars, 5 μ m. Correlations between the numbers of EET-forming and total eosinophils (B), and the EPX degranulation score (C). Bars, 10 μ m. *, $P < 0.05$; ****, $P < 0.0001$.

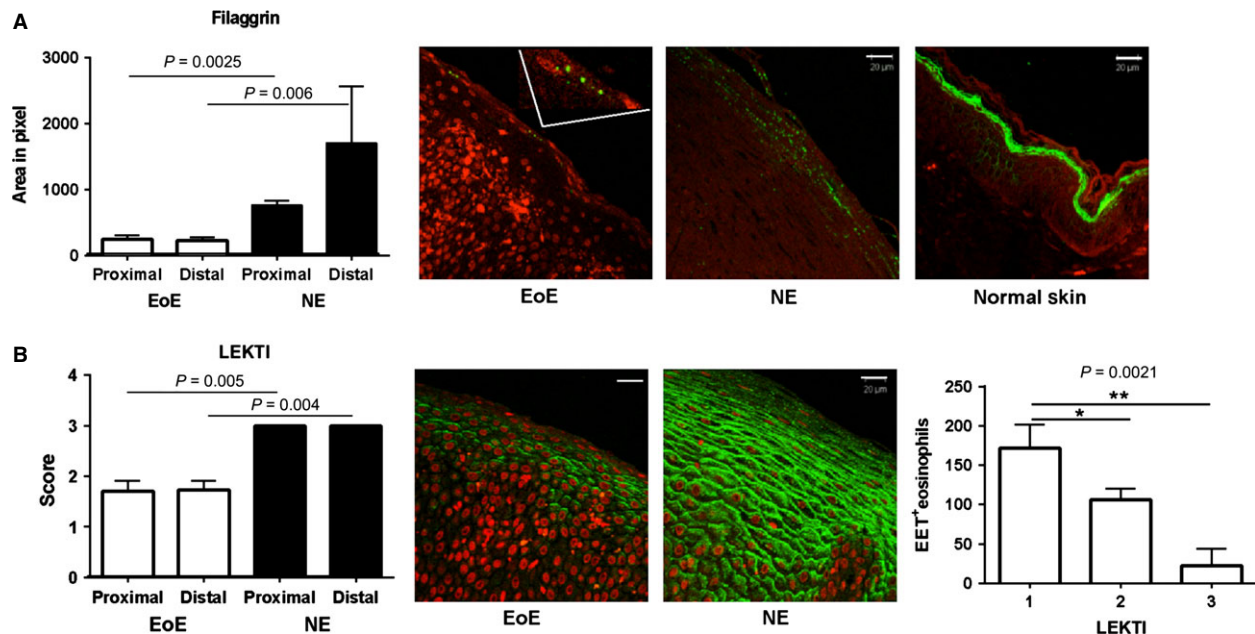


Figure 2 Decreased expression of filaggrin (A) and LEKTI (B) in active EoE ($n = 10$), as compared to normal esophagus (NE, $n = 3$). Filaggrin is expressed in single cells in EoE (insert) as compared to a linear expression in NE and skin. LEKTI is expressed in the apical

part of the epithelium in NE, but declined in active EoE, inversely correlating with the number of EET-forming eosinophils. Nuclei were stained with propidium iodide. Bars, 20 μm . *, $P < 0.05$; **, $P < 0.01$.

EET-positive eosinophils ($P = 0.0021$) (Fig. 2B; Table S2 in Supporting Information).

The expression of antimicrobial peptides is increased in active EoE

As a decreased production of antimicrobial peptides due to the Th2 cytokine milieu has been reported in AD (23, 24), we investigated whether the expression is altered in EoE. Interestingly, compared with NE, the expression of LL-37, HBD-2, HBD-3, and psoriasin was significantly higher in EoE (Fig. 3A–D). Moreover, a significant inverse correlation was found between LEKTI and HBD-3 ($r = -0.75$; $P = 0.0005$) or LL-37 ($r = -0.55$; $P = 0.022$), respectively. When we related antimicrobial peptide expression and numbers of EET-positive eosinophils, a significant positive correlation was found for psoriasin ($r = 0.55$; $P = 0.016$), but not for LL-37, HBD-2, or HBD-3 (see Table S2 in Supporting Information).

The expression of epithelial cytokines is increased in active EoE

Environmental factors, such as microbes, allergens, and irritants, passing the defective epithelial barrier may lead to an activation of epithelial cells, resulting in the infiltration of inflammatory cells and cytokine release (13, 14). TSLP has been reported to be expressed by epithelial cells in AD (16) as well as EoE (31), and to directly stimulate eosinophils to form EETs *in vitro* (6). In all EoE biopsies, we observed

TSLP expression predominantly in the apical parts of the epithelium (Fig. 4A). TSLP expression significantly correlated with the numbers of EET-positive eosinophils ($r = 0.52$; $P = 0.02$), suggesting a direct effect of TSLP on eosinophils for the production of EETs (see Table S2 in Supporting Information).

IL-25, a member of the IL-17 cytokine family, was shown to be released by epithelial cells upon microbial and allergen protease stimulation and to promote Th2 inflammation (18, 32). In EoE, IL-25 was extensively expressed by epithelial cells, but was absent in normal esophageal tissue (proximal $P = 0.0006$, distal $P = 0.0005$) (Fig. 4B). Interestingly, the number of IL-25-expressing epithelial cells negatively correlated with the numbers of EET-positive eosinophils ($r = -0.6$; $P = 0.013$) (see Table S2 in Supporting Information). Infiltrating IL-25-positive cells were rarely detected in EoE with mean numbers of 2.5 and 10 cells/ mm^2 in the epithelium and subepithelium, respectively.

IL-32 expression has been demonstrated in lesional AD skin, chronic rhinosinusitis, as well as in bronchial endothelial cells upon stimulation with IFN- γ , Th1 cells, and rhinovirus (33–35). In chronic rhinosinusitis, IL-32 was expressed by epithelial cells as well as by inflammatory cells (35). In EoE biopsies, we detected IL-32 expression by both epithelial and infiltrating cells. Approximately 75% of epithelial cells expressed IL-32. Furthermore, we observed IL-32-positive infiltrating cells in specimens of EoE, but not in NE (Fig. 4C). IL-32 expression did not correlate with the numbers of EET-positive eosinophils in EoE biopsies (see Table S2 in Supporting Information).

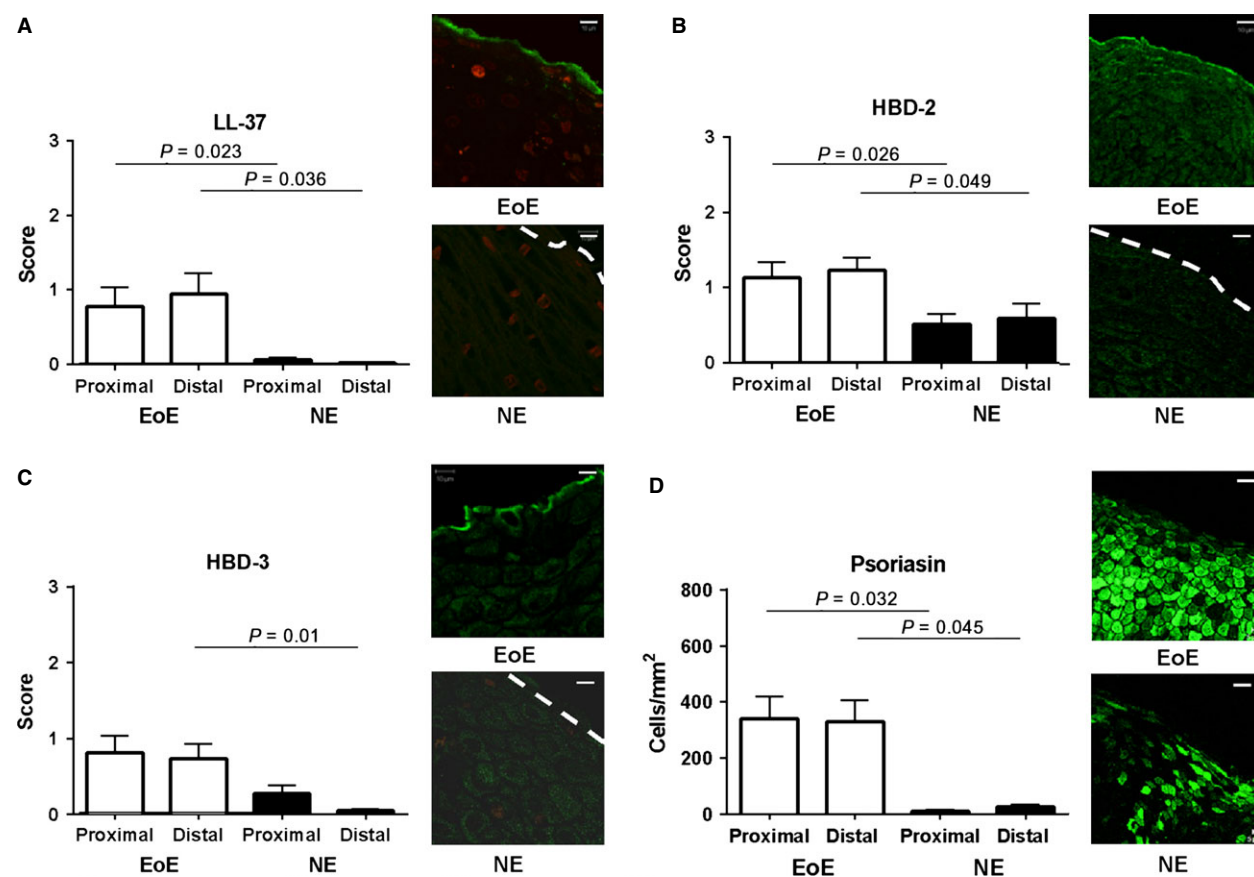


Figure 3 Increased expression of cathelicidin LL-37 (A), human beta-defensin (HBD)-2 (B), HBD-3 (C), and psoriasin (D) in active EoE ($n = 10$) as compared to normal esophagus (NE, $n = 3$). Representative images show a bandlike expression of cathelicidin LL-37,

HBD-2, and HBD-3 at the epithelial surface in EoE but not NE, and an extensive vs focal epithelial psoriasin expression in EoE and NE, respectively. Bars, 10 μ m.

IL-33, an alarmin expressed by barrier tissue cells, was shown to play a pathogenic role in allergic airway and skin diseases (36). In airway epithelial cells, IL-33 is constitutively stored in the nuclei, but translocated to the cytoplasm and released upon exposure to an environmental fungus (37). In NE tissues, nuclear expression of IL-33 was noted in epithelial cells, but was nearly absent in the cytoplasm. In EoE, a significantly increased nuclear and cytoplasmic expression of IL-33 was observed in comparison with NE (nuclear, $P = 0.013$; cytoplasmic, $P = 0.02$) (Fig. 4D, E). In agreement with previous observations, nuclear expression of IL-33 was predominantly in the basal cell layer of the epithelium (37). IL-33 expression did not correlate with EET numbers in EoE biopsies (see Table S2 in Supporting Information).

Little evidence for an IL-17-driven process in active EoE

As IL-17 has been shown to be involved in defense to microbes as well as allergic diseases (22), we investigated whether IL-17 is expressed in EoE. We detected few IL-17-

positive cells (mean 15 cells/mm²) (see Fig. S2A, in Supporting Information), mainly IL-17-positive mast cells, but no IL-17-positive T cells in EoE tissue (see Fig. S2B in Supporting Information). Furthermore, the expression of IL-23, a regulator of Th17 cells (38), was detected in single dendritic cells in the subepithelium only (mean 4 cells/mm²), but not in the esophageal epithelium. IL-23 expression by epithelial cells was not observed.

The formation of eosinophil extracellular traps correlates with white exudates

Next, we asked whether the number of EET-positive eosinophils and total number of infiltrating eosinophils correlated with clinical and endoscopic findings. We observed more infiltrating eosinophils and EET-positive eosinophils in EoE specimens from patients with a history of food impactions and concomitant allergic diseases as compared with those without. The differences in the mean numbers were significant for the total number of eosinophils ($P = 0.017$; $P = 0.04$), but were not significantly different for

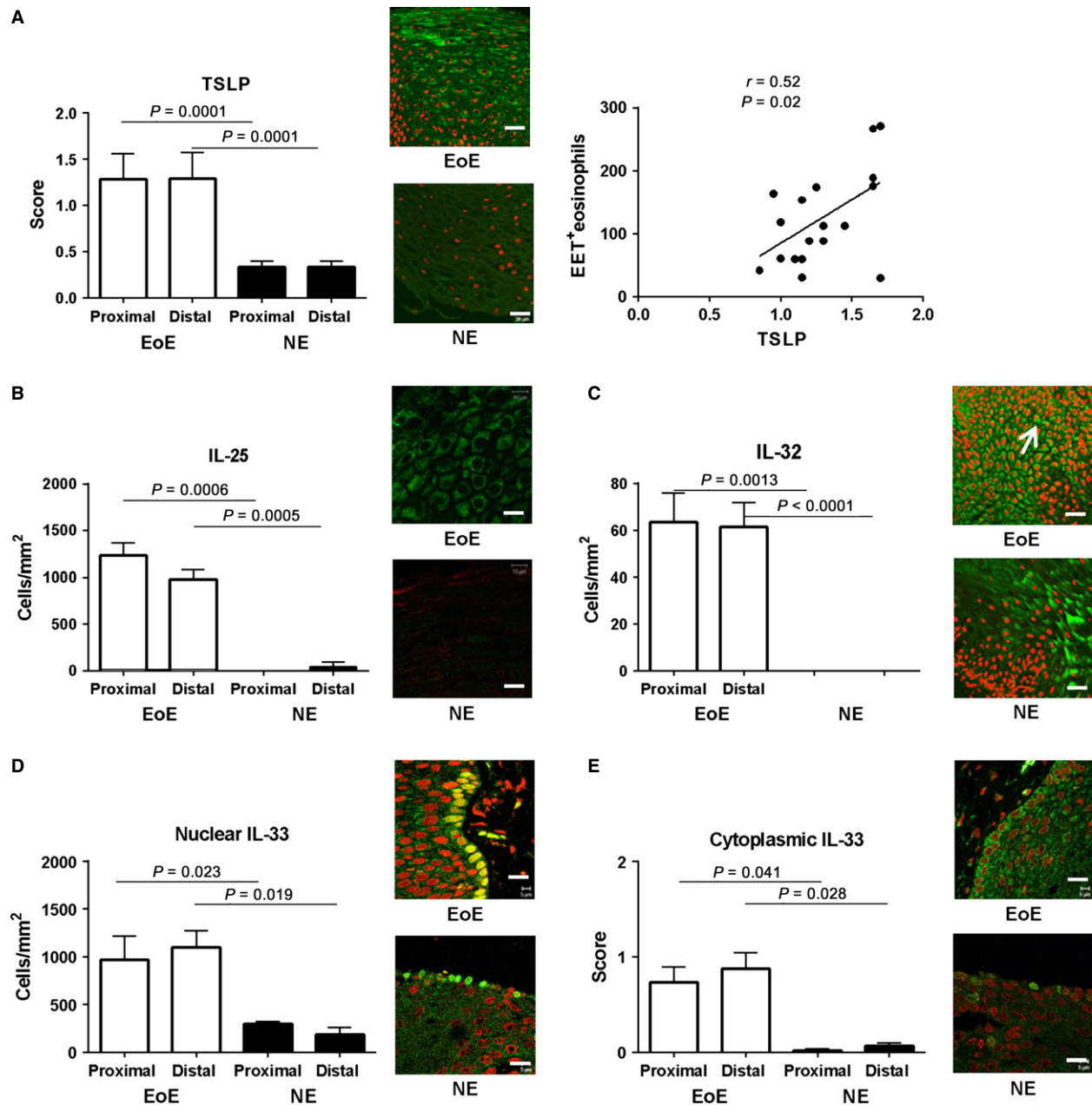


Figure 4 Increased expression of TSLP (A), IL-25 (B), IL-32 (C), nuclear (D), and cytoplasmic IL-33 (E) in active EoE ($n = 10$) as compared to normal esophagus (NE, $n = 3$). TSLP expression correlates with EET-positive eosinophils (panel A). Representative images illustrate TSLP, IL-25, and IL-33 expression. IL-32 expres-

sion is shown in epithelial cells and in infiltrating cells (arrow). Nuclear IL-33 expression is largely restricted to basal epithelial cells, in both EoE and NE. Nuclei were stained with propidium iodide. Bars, 5 μm (panels D and E), 10 μm (panel B), and 20 μm (panels A and C).

EET-positive eosinophils ($P = 0.50$; $P = 0.75$) (Fig. 5A). For the symptom, chest pain, no correlation with EET-positive eosinophils or eosinophil numbers was found (total number of eosinophils, $P = 0.29$; EET-positive eosinophils, $P = 0.36$). Looking at endoscopic findings, the mean levels of EET-positive eosinophils and total eosinophils increased with the presence and extent of white exudates (Fig. 5B). Increased

numbers of infiltrating eosinophils were found in biopsies from patients with high numbers of corrugated rings ($P = 0.003$). The number of EET-positive eosinophils tended to be higher in patients with strictures ($P = 0.056$). When we related the individual number of EET-positive eosinophils with endoscopic findings, we found a correlation with the frequency of white exudates ($r = 0.61$; $P = 0.007$), but not for

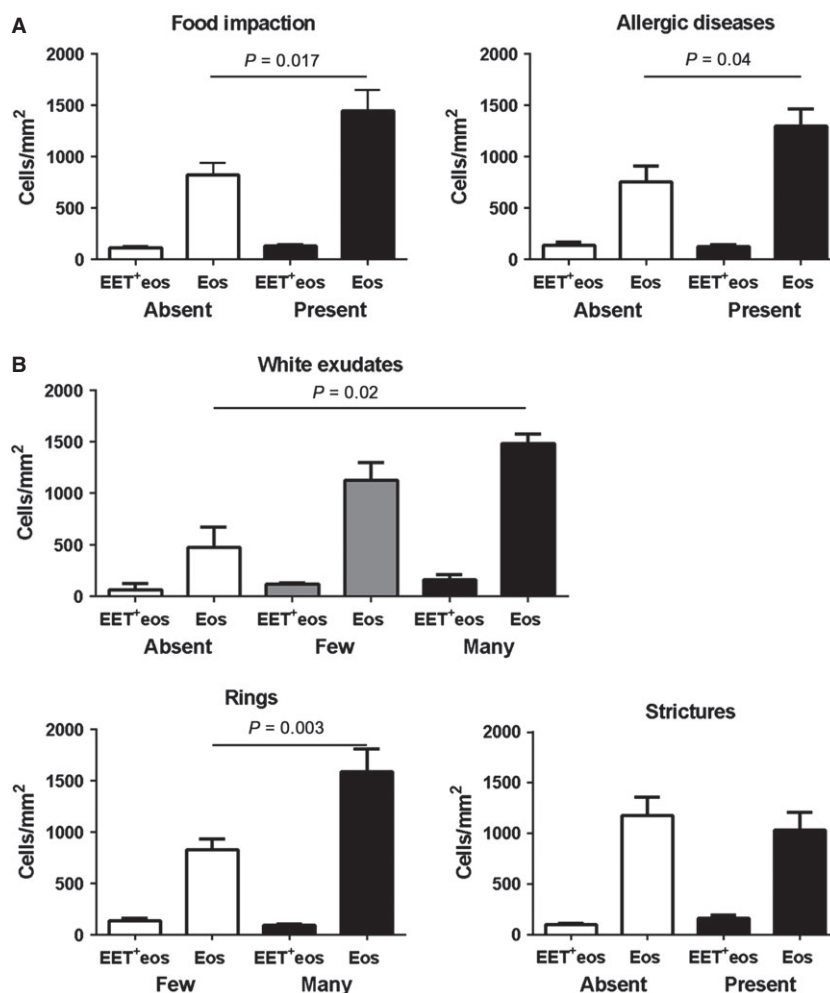


Figure 5 Eosinophil numbers and EET formation show correlations with some clinical and endoscopic findings. Bar graphs illustrate that the number of infiltrating esophageal eosinophils is signifi-

cantly higher in EoE patients with food impaction and additional allergic diseases (A) and with white exudates and rings (B), but not in EoE patients with strictures (B).

the presence of strictures ($r = 0.44$; $P = 0.07$) (see Table S2 in Supporting Information).

Discussion

This study demonstrates that eosinophils infiltrating the inflamed esophagus of EoE patients form EETs consisting of DNA nets binding eosinophil granule proteins. EET formation was observed in association with defects of the epithelial barrier as well as with increased production of antimicrobial peptides and epithelial-derived cytokines which are known to drive Th2 inflammation and eosinophil activation. Moreover, the number of EET-positive eosinophils correlated significantly with the degree of eosinophil infiltration and degranulation as well as with the endoscopic finding of white exudates.

Having detected EETs in active EoE, we raised the question of which factors might predispose to eosinophils forming

EETs in the esophagus. There is increasing evidence that a structural and functional impairment of the epithelial barrier plays a pathogenic role in EoE (12, 13). In agreement with recent studies reporting a downregulation of the filaggrin gene and its protein expression in EoE (39, 40), we observed significantly decreased filaggrin expression in active EoE as compared to NE. However, it should be noted that filaggrin expression is dramatically diminished in the esophagus compared to skin, suggesting that its downregulation in EoE may not really contribute to a potential epithelial barrier defect. On the other hand, the expression of the protease inhibitor LEKTI, usually present in all stratified epithelia (41), was greatly reduced in active EoE as compared to NE and its expression correlated inversely with EET numbers. Thus, it seems likely that a decrease in LEKTI results in a defective epithelial barrier, thus enabling bacteria to penetrate and directly to stimulate eosinophils to form EETs as shown *in vitro* for *Staphylococcus aureus* (6).

It has been demonstrated previously that a lack of LEKTI expression in keratinocytes results in increased activity of the protease kallikrein 5, thus directly activating proteinase-activated receptor (PAR)-2 which in turn triggers subsequent TSLP expression and Th2-type skin inflammation (42, 43). In active EoE, we found an increased production of TSLP associated with low LEKTI expression, in agreement with these earlier findings. Moreover, the expression of TSLP significantly correlated with EET formation in EoE tissue samples, suggesting that pathogen-mediated and epithelial cell-derived TSLP directly activates the infiltrating eosinophils to form EETs. This conclusion is supported by the observation that TSLP also activates eosinophils to form EETs *in vitro* (6).

We also observed in active EoE, as compared to NE, an increased epithelial expression of IL-25, IL-32, and IL-33, all of which are known to promote Th2 inflammation. IL-25 has been reported to enhance eosinophil survival and upregulate adhesion molecule, chemokine, and IL-6 expression by eosinophils (44, 45). Based on our results, a low expression of IL-25 in epithelial cells was associated with a high number of EET-positive eosinophils in EoE tissues, suggesting that IL-25 is not the trigger responsible for EET formation. IL-32 can be produced by various cell types, including infiltrating inflammatory and epithelial cells. IL-32, produced by epithelial cells upon stimulation with pro-inflammatory cytokines in synergism with the NOD-like receptors activated by pathogen-associated molecular patterns (PAMPs), for example, bacterial infection, was shown to increase the expression of adhesion molecules and cytokines by eosinophils as well as the formation of EETs (46). However, under the *in vivo* conditions reflected by this study, there was no obvious correlation between IL-32 expression and EET formation, suggesting that the contribution of IL-32 to the formation of EETs is small or indirect.

In NE, we detected nuclear IL-33 expression only. In EoE epithelium, however, both nuclear and cytoplasmic IL-33 expression could be observed. IL-33 has been shown to be constitutively expressed in the nuclei of epithelial cells, in particular in the basal cell layer, but it appeared in the cytoplasm upon allergen exposure (37). Therefore, these data support the concept that epithelial cells are activated in EoE. Interestingly, direct activation of eosinophils by IL-33 enhanced their superoxide anion production, degranulation, and cytokine production (47, 48). However, as with IL-32, we observed no significant correlation between IL-33 expression and the formation of EETs. Taken together, although IL-25, IL-32, and IL-33 exhibit increased expression in esophageal epithelial cells in active EoE and have previously been shown to be able to activate eosinophils under *in vitro* conditions, there is little evidence that these cytokines play a major role in EET formation *in vivo*. However, they may contribute to EoE pathogenesis through other pathways.

Compared with NE, we observed an increased expression of antimicrobial peptides such as LL-37, HBD-2 and HBD-3, and psoriasin in active EoE. In contrast, a decreased expression of HBD-1 and HBD-3 has been reported in pediatric

EoE (49). High values for EET-positive eosinophils were associated with increased antimicrobial peptide expression. We hypothesize that these findings are a consequence of an altered epithelial barrier function, a conclusion which is supported by the observation that low LEKTI expression correlated not just with EET formation, but also with increased antimicrobial peptide expression, particularly HBD-3 and LL-37. In agreement with a previous study, we found only minimal expression of IL-17, a cytokine that is known to stimulate antimicrobial peptide expression by epithelial cells (50, 51).

What might be the purpose of EET formation in active EoE? The presence of EETs in infectious diseases and their *in vitro* bacterial killing effects have suggested that EETs play a role in host defense to microbes (5, 6, 8). Moreover, *Staphylococcus aureus* directly stimulates eosinophils to generate EETs, which in turn are able to kill the bacteria (6). A pathogen responsible for EET formation in EoE has not been identified yet, but the observation of an impaired barrier function implies that microbes colonizing the epithelial surface of the esophagus might be causative agents. Colonization and infection with *Candida albicans* has been observed in EoE patients under corticosteroid therapy (52). The association of *Helicobacter pylori* infection with eosinophil infiltration of the esophagus has been discussed controversially (53–55). Proton pump inhibitors (PPI), which are used for the treatment of EoE, have been reported to alter the bacterial composition in the esophagus (56). Whether the microbiota of EoE differs from that of the normal esophagus remains to be investigated.

The formation of EETs is an active process and requires an adequate stimulation of eosinophils by microbes, cytokines, or complement factor C5a (5). In addition to EETs, we observed intense granule deposition in EoE tissues confirming previous observations (25, 57). With electron microscopy, these extracellular granules have been clearly identified outside of eosinophils (58). Extracellular granule deposits off to the side of and distinct from extracellular traps have also been reported for eosinophilic skin diseases (8), suggesting that granule release and the formation of EETs occur in parallel following the activation of eosinophils. Extensive eosinophil degranulation has previously been described as a characteristic feature of EoE, distinguishing it from gastroesophageal reflux disease (57).

Taken together, our results provide further evidence that the epithelial barrier function of the esophagus is impaired in active EoE, thus promoting and/or perpetuating eosinophil inflammation. We postulate the following hypothesis: Due to an defective epithelial barrier, particularly owing to the expression of protease inhibitors (e.g., LEKTI), microbes are able to penetrate into the epithelium and to initiate an inflammatory response. To defend against invading pathogens, epithelial cells would enhance the production of antimicrobial peptides and release cytokines, such as TSLP, IL-25, IL-32, and IL-33 followed by an accumulation and activation of eosinophils. By forming EETs, eosinophils are thus able to kill microbes and, in consequence, to form a secondary defense barrier. Whether this hypothesis could be transferred

to other esophageal conditions characterized by epithelial barrier defects and eosinophils, for example, GERD, has to be proven in further studies.

Acknowledgments

We thank Monica Schillizzi, Nathalie Friedli, Florence Charriere, Matthias Stuck, and Evelyn Kozlowski for their competent technical assistance.

Funding

Supported by the Swiss National Science Foundation (SNF) with grants to H.U.S. (Grant number 310030_146181) and S.Y. (Grant number 310030_146215). S.R.H. participates in a MD-PhD program funded by the SNF.

References

1. Straumann A, Aceves SS, Blanchard C, Collins MH, Furuta GT, Hirano I et al. Pediatric and adult eosinophilic esophagitis: similarities and differences. *Allergy* 2012;**67**:477–490.
2. Simon D, Straumann A, Dahinden C, Simon HU. Frequent sensitization to *Candida albicans* and profilins in adult eosinophilic esophagitis. *Allergy* 2013;**68**:945–948.
3. Simon D, Marti H, Heer P, Simon HU, Braathen LR, Straumann A. Eosinophilic esophagitis is frequently associated with IgE-mediated allergic airway diseases. *J Allergy Clin Immunol* 2005;**115**:1090–1092.
4. Straumann A, Bauer M, Fischer B, Blaser K, Simon HU. Idiopathic eosinophilic esophagitis is associated with a T(H)2-type allergic inflammatory response. *J Allergy Clin Immunol* 2001;**108**:954–961.
5. Yousefi S, Gold JA, Andina N, Lee JJ, Kelly AM, Kozlowski E et al. Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. *Nat Med* 2008;**14**:949–953.
6. Morshed M, Yousefi S, Stöckle C, Simon HU, Simon D. Thymic stromal lymphopoietin stimulates the formation of eosinophil extracellular traps. *Allergy* 2012;**67**:1127–1137.
7. Simon D, Simon HU, Yousefi S. Extracellular DNA traps in allergic, infectious, and autoimmune diseases. *Allergy* 2013;**68**:409–416.
8. Simon D, Hoesli S, Roth N, Staedler S, Yousefi S, Simon HU. Eosinophil extracellular DNA traps in skin diseases. *J Allergy Clin Immunol* 2011;**127**:194–199.
9. Dworski R, Simon HU, Hoskins A, Yousefi S. Eosinophil and neutrophil extracellular DNA traps in human allergic asthmatic airways. *J Allergy Clin Immunol* 2011;**127**:1260–1266.
10. Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet* 2006;**38**:441–446.
11. de Boer WI, Sharma HS, Baelemans SM, Hoogsteden HC, Lambrecht BN, Braunstahl GJ. Altered expression of epithelial junctional proteins in atopic asthma: possible role in inflammation. *Can J Physiol Pharmacol* 2008;**86**:105–112.
12. Sherrill JD, Kc K, Wu D, Djukic Z, Caldwell JM, Stucke EM et al. Desmoglein-1 regulates esophageal epithelial barrier function and immune responses in eosinophilic esophagitis. *Mucosal Immunol* 2014;**7**:718–729.
13. Katzka DA, Tadi R, Smyrk TC, Katarya E, Sharma A, Geno DM et al. Effects of topical steroids on tight junction proteins and spongiosis in esophageal epithelia of patients with eosinophilic esophagitis. *Clin Gastroenterol Hepatol* 2014;**12**:1824–1829. Epub ahead of print.
14. Lambrecht BN, Hammad H. The airway epithelium in asthma. *Nat Med* 2012;**18**:684–692.
15. Kuo IH, Yoshida T, De Benedetto A, Beck LA. The cutaneous innate immune response in patients with atopic dermatitis. *J Allergy Clin Immunol* 2013;**131**:266–278.
16. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 2002;**3**:673–680.
17. Pecaric-Petkovic T, Didichenko SA, Kaempfer S, Spiegel N, Dahinden CA. Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. *Blood* 2009;**113**:1526–1534.
18. Angkasekwinai P, Park H, Wang YH, Wang YH, Chang SH, Corry DB et al. Interleukin 25 promotes the initiation of proallergic type 2 responses. *J Exp Med* 2007;**204**:1509–1517.
19. Wang YH, Angkasekwinai P, Lu N, Voo KS, Arima K, Hanabuchi S et al. IL-25 augments type 2 immune responses by enhancing the expansion and functions of TSLP-DC-activated Th2 memory cells. *J Exp Med* 2007;**204**:1837–1847.
20. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 2005;**23**:479–490.
21. Howell MD, Kim BE, Gao P, Grant AV, Boguniewicz M, De Benedetto A et al. Cytokine modulation of atopic dermatitis filaggrin skin expression. *J Allergy Clin Immunol* 2007;**120**:150–155.
22. Eyerich K, Pennino D, Scarponi C, Foerster S, Nasorri F, Behrendt H et al. IL-17 in atopic eczema: linking allergen-specific adaptive and microbial-triggered innate immune response. *J Allergy Clin Immunol* 2009;**123**:59–66.
23. Howell MD, Boguniewicz M, Pastore S, Novak N, Bieber T, Girolomoni G et al. Mechanism of HBD-3 deficiency in atopic dermatitis. *Clin Immunol* 2006;**121**:332–338.
24. Howell MD, Gallo RL, Boguniewicz M, Jones JF, Wong C, Streib JE et al. Cytokine milieu of atopic dermatitis skin subverts the innate immune response to vaccinia virus. *Immunity* 2006;**24**:341–348.
25. Straumann A, Hoesli S, Bussmann Ch, Stuck M, Perkins M, Collins LP et al. Anti-eosinophil activity and clinical efficacy of the CRTH2 antagonist OC000459 in eosinophilic esophagitis. *Allergy* 2013;**68**:375–385.
26. Roth N, Städler S, Lemann M, Hösli S, Simon HU, Simon D. Distinct eosinophil cytokine expression patterns in skin diseases - the possible existence of functionally different eosinophil subpopulations. *Allergy* 2011;**66**:1477–1486.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The arrangement of eosinophils in EoE tissues.

Figure S2. The expression of IL-17 in EoE.

Table S1. Patient characteristics.

Table S2. Cross-correlations among different parameters in active EoE patients: Number of EET positive eosinophils, epidermal barrier markers, local expression of antimicrobial peptides and cytokines in the epithelium, and endoscopic findings.

27. Weidinger S, Illig T, Baurecht H, Irvine AD, Rodriguez E, Diaz-Lacava A et al. Loss-of-function variations within the filaggrin gene predispose for atopic dermatitis with allergic sensitizations. *J Allergy Clin Immunol* 2006;**118**:214–219.
28. Palmer CN, Ismail T, Lee SP, Terron-Kwiatkowski A, Zhao Y, Liao H et al. Filaggrin null mutations are associated with increased asthma severity in children and young adults. *J Allergy Clin Immunol* 2007;**120**:64–68.
29. Chavanas S, Bodemer C, Rochat A, Hamel-Teillac D, Ali M, Irvine AD et al. Mutations in SPINK5, encoding a serine protease inhibitor, cause Netherton syndrome. *Nat Genet* 2000;**25**:141–142.
30. Walley AJ, Chavanas S, Moffatt MF, Esnouf RM, Ubhi B, Lawrence R et al. Gene polymorphism in Netherton and common atopic disease. *Nat Genet* 2001;**29**:175–178.
31. Noti M, Wojno ED, Kim BS, Siracusa MC, Giacomini PR, Nair MG et al. Thymic stromal lymphopoietin-elicited basophil responses promote eosinophilic esophagitis. *Nat Med* 2013;**19**:1005–1013.
32. Kouzaki H, Tojima I, Kita H, Shimizu T. Transcription of interleukin-25 and extracellular release of the protein is regulated by allergen proteases in airway epithelial cells. *Am J Respir Cell Mol Biol* 2013;**49**:741–750.
33. Meyer N, Zimmermann M, Bürgler S, Bassin C, Woehrl S, Moritz K et al. IL-32 is expressed by human primary keratinocytes and modulates keratinocyte apoptosis in atopic dermatitis. *J Allergy Clin Immunol* 2010;**125**:858–865.
34. Meyer N, Christoph J, Makrinioti H, Indermitte P, Rhyner C, Soyka M et al. Inhibition of angiogenesis by IL-32: possible role in asthma. *J Allergy Clin Immunol* 2012;**129**:964–973.
35. Keswani A, Chustz RT, Suh L, Carter R, Peters AT, Tan BK et al. Differential expression of interleukin-32 in chronic rhinosinusitis with and without nasal polyps. *Allergy* 2012;**67**:25–32.
36. Ohno T, Morita H, Arae K, Matsumoto K, Nakae S. Interleukin-33 in allergy. *Allergy* 2012;**67**:1203–1214.
37. Kouzaki H, Iijima K, Kobayashi T, O'Grady SM, Kita H. The danger signal, extracellular ATP, is a sensor for an airborne allergen and triggers IL-33 release and innate Th2-type responses. *J Immunol* 2011;**186**:4375–4387.
38. Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem* 2003;**278**:1910–1914.
39. Blanchard C, Stucke EM, Burwinkel K, Caldwell JM, Collins MH, Ahrens A et al. Coordinate interaction between IL-13 and epithelial differentiation cluster genes in eosinophilic esophagitis. *J Immunol* 2010;**184**:4033–4041.
40. Matoso A, Mukkada VA, Lu S, Monahan R, Cleveland K, Noble L et al. Expression microarray analysis identifies novel epithelial-derived protein markers in eosinophilic esophagitis. *Mod Pathol* 2013;**26**:665–676.
41. Bitoun E, Micheloni A, Lamant L, Bonnart C, Tartaglia-Polcini A, Cobbold C et al. LE-KTI proteolytic processing in human primary keratinocytes, tissue distribution and defective expression in Netherton syndrome. *Hum Mol Genet* 2003;**12**:2417–2430.
42. Briot A, Deraison C, Lacroix M, Bonnart C, Robin A, Besson C et al. Kallikrein 5 induces atopic dermatitis-like lesions through PAR2-mediated thymic stromal lymphopoietin expression in Netherton syndrome. *J Exp Med* 2009;**206**:1135–1147.
43. Kouzaki H, O'Grady SM, Lawrence CB, Kita H. Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. *J Immunol* 2009;**183**:1427–1434.
44. Wong CK, Cheung PF, Ip WK, Lam CW. Interleukin-25-induced chemokines and interleukin-6 release from eosinophils is mediated by p38 mitogen-activated protein kinase, c-Jun N-terminal kinase, and nuclear factor-kappaB. *Am J Respir Cell Mol Biol* 2005;**33**:186–194.
45. Cheung PF, Wong CK, Ip WK, Lam CW. IL-25 regulates the expression of adhesion molecules on eosinophils: mechanism of eosinophilia in allergic inflammation. *Allergy* 2006;**61**:878–885.
46. Wong CK, Dong J, Lam CW. Molecular mechanisms regulating the synergism between IL-32 γ and NOD for the activation of eosinophils. *J Leukoc Biol* 2014;**95**:631–642.
47. Cherry WB, Yoon J, Bartemes KR, Iijima K, Kita H. A novel IL-1 family cytokine, IL-33, potentially activates human eosinophils. *J Allergy Clin Immunol* 2008;**121**:1484–1490.
48. Stolarski B, Kurowska-Stolarska M, Kewin P, Xu D, Liew FY. IL-33 exacerbates eosinophil-mediated airway inflammation. *J Immunol* 2010;**185**:3472–3480.
49. Schroeder S, Robinson ZD, Masterson JC, Hosford L, Moore W, Pan Z et al. Esophageal human β -defensin expression in eosinophilic esophagitis. *Pediatr Res* 2013;**73**:647–654.
50. Blanchard C1, Stucke EM, Rodriguez-Jimenez B, Burwinkel K, Collins MH, Ahrens A et al. A striking local esophageal cytokine expression profile in eosinophilic esophagitis. *J Allergy Clin Immunol* 2011;**127**:208–217.
51. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* 2006;**203**:2271–2279.
52. Straumann A, Conus S, Degen L, Felder S, Kummer M, Engel H et al. Budesonide is effective in adolescent and adult patients with active eosinophilic esophagitis. *Gastroenterology* 2010;**139**:1526–1537.
53. Kalach N, Huvenne H, Gosset P, Papadopoulos S, Dehecq E, Decoster A et al. Eosinophil counts in upper digestive mucosa of Western European children: variations with age, organs, symptoms, *Helicobacter pylori* status, and pathological findings. *J Pediatr Gastroenterol Nutr* 2011;**52**:175–182.
54. Dellon ES, Peery AF, Shaheen NJ, Morgan DR, Hurrell JM, Lash RH et al. Inverse association of esophageal eosinophilia with *Helicobacter pylori* based on analysis of a US pathology database. *Gastroenterology* 2011;**141**:1586–1592.
55. Ronkainen J, Talley NJ, Aro P, Storskrubb T, Johansson SE, Lind T et al. Prevalence of oesophageal eosinophils and eosinophilic oesophagitis in adults: the population-based Kalixanda study. *Gut* 2007;**56**:615–620.
56. Amir I, Konikoff FM, Oppenheim M, Gophna U, Half EE. Gastric microbiota is altered in oesophagitis and Barrett's oesophagus and further modified by proton pump inhibitors. *Environ Microbiol* 2014;**16**:2905–2914.
57. Protheroe C, Woodruff SA, de Petris G, Mukkada V, Ochkur SI, Janarthanan S et al. A novel histologic scoring system to evaluate mucosal biopsies from patients with eosinophilic esophagitis. *Clin Gastroenterol Hepatol* 2009;**7**:749–755.
58. Saffari H, Hoffman LH, Peterson KA, Fang JC, Leiferman KM, Pease LF 3rd et al. Electron microscopy elucidates eosinophil degranulation patterns in patients with eosinophilic esophagitis. *J Allergy Clin Immunol* 2014;**133**:1728–1734.

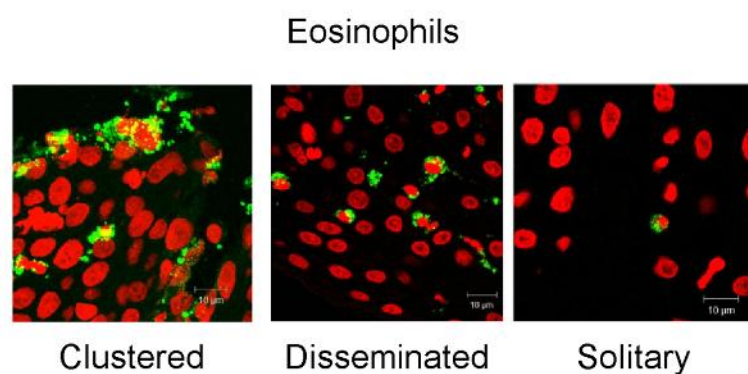


Figure S1 The arrangement of eosinophils in EoE tissues. Eosinophils were arranged in clusters (left), as disseminated (middle), or solitary individuals (right). Eosinophils were identified using anti-EPX mAb. Nuclei were stained with propidium iodide. Bars, 10 mm.

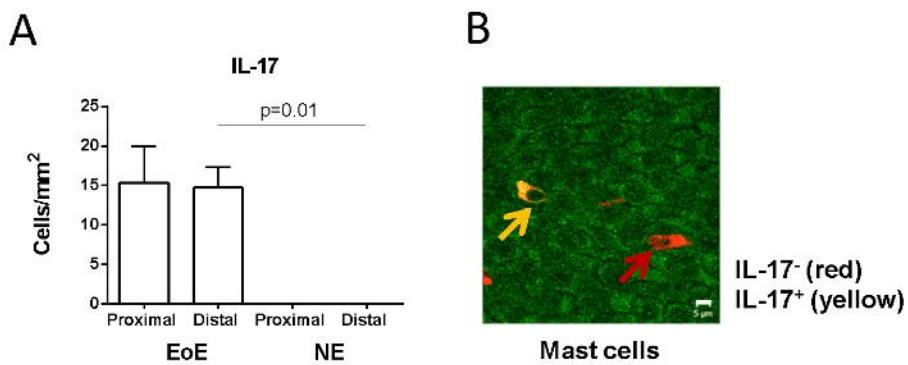


Figure S2 The expression of IL-17 in EoE. Left: A bar graph shows the number of IL-17-expressing cells in EoE and normal esophagus, for both proximal and distal biopsies. Right: Representative original image illustrating two mast cells, one expressing IL-17 (yellow), the other not (red). Mast cells were identified using anti-mast cell tryptase Ab. Bar, 5 mm.

Supplementary Table S1

Patient characteristics

Pat- ient	Gen- der	Age	Age at EoE diag- nosis	EoE dura- tion	Atopic diseases			FA	IgE	Endoscopy					Histology			GERD	PPI
		years	years	years	Asthma	Allergic rhinitis	AD		kU/l	Edema	Furrows	Exudates white	Rings	Strictures*	Peak Eos	Eos load	Eos absce- sses		
1	m	51	49	10	no	no	no	no	30	yes	yes	yes	yes	no	210	174	yes	no	no
2	m	48	36	31	yes	no	no	no	256	yes	yes	yes	yes	no	261	208	no	no	no
3	m	36	32	20	yes	yes	no	yes	148	yes	yes	yes	yes	yes	123	84	no	no	no
4	m	39	32	10	no	yes	no	no	85	yes	yes	yes	yes	yes	217	156	yes	no	no
5	m	43	28	17	yes	yes	yes	no	76	yes	yes	yes	yes	no	357	269	yes	no	yes
6	m	59	56	18	yes	yes	yes	no	187	yes	yes	yes	yes	no	262	177	yes	no	no
7	m	53	48	6	no	no	no	no	26	yes	yes	yes	yes	no	98	65	no	no	no
8	m	28	24	10	no	no	no	no	532	yes	yes	yes	yes	no	87	56	no	no	no
9	f	25	22	6	yes	yes	yes	no	1643	yes	yes	yes	yes	no	114	60	yes	no	no
10	m	50	33	19	yes	no	no	no	51	yes	yes	yes	yes	yes	89	55	yes	no	no
11	m	54	53	11	yes	no	no	no	n.d.	yes	yes	yes	yes	no	233	168	yes	yes	no
12	m	69	66	25	no	no	no	no	42	yes	yes	yes	yes	no	111	58	no	no	no
13	f	33	30	20	yes	yes	no	no	67	yes	yes	yes	yes	no	247	205	yes	no	no
14	m	41	35	16	yes	yes	no	no	177	yes	yes	yes	yes	no	250	167	no	no	no
15	m	58	55	10	yes	yes	yes	no	88	yes	yes	yes	yes	no	72	52	no	no	no
16	m	43	41	3	yes	yes	no	no	1546	yes	yes	no	yes	no	101	65	no	no	no
17	m	61	55	20	no	no	no	no	18	yes	yes	yes	no	no	256	192	yes	no	no
18	m	41	38	16	yes	yes	yes	yes	71	yes	yes	yes	yes	no	136	108	no	no	no

AD, atopic dermatitis; FA, food allergy; IgE, immunoglobulin E; GERD, Gastro-esophageal reflux disease; PPI, proton pump inhibitor

*Strictures were reported if the esophagus could be passed with a standard endoscope (9 mm outer diameter) with some resistance, often inducing lacerations, or a 6-mm outer diameter endoscope, and if it could not be passed with a 6-mm outer diameter endoscope.

Supplementary Table S2 Cross correlations among different parameters in active EoE patients: Number of EET positive eosinophils, epidermal barrier markers, local expression of antimicrobial peptides and cytokines in the epithelium, and endoscopic findings.

		Epithelial barrier		Antimicrobial peptides				Epithelial cytokines					Endoscopic findings		
		Filaggrin	LEKTI	LL-37	HBD-2	HBD-3	Psoriasin S100A7	TSLP	IL-25	IL-32	IL-33 nuclear	IL-33 cytoplasmic	White exudates	Rings	Strictures
EETs	r	-0.068	-0.56	0.39	0.25	0.18	0.55	0.52	-0.6	0.13	0.11	-0.4	0.61	-0.22	0.44
	P	0.79	0.016	0.12	0.13	0.5	0.016	0.02	0.013	0.8	0.84	0.43	0.007	0.38	0.07
Filaggrin	r		0.49	-0.28	-0.02	-0.26	-0.17	0.33	-0.32	n.d.	n.d.	n.d.	-0.05	0.37	-0.27
	P		0.038	0.27	0.94	0.31	0.5	0.41	0.23	n.d.	n.d.	n.d.	0.84	0.13	0.28
LEKTI	r	0.49		-0.55	-0.4	-0.75	-0.45	-0.71	0.46	n.d.	n.d.	n.d.	-0.37	0.54	-0.50
	P	0.038		0.022	0.07	0.0005	0.06	0.047	0.07	n.d.	n.d.	n.d.	0.13	0.02	0.033

Significant values for Pearson's coefficient (r) and P are indicated in red.