

Epithelial origin of eosinophilic esophagitis



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Eosinophilic esophagitis (EoE) is a chronic, allergen-driven inflammatory disease of the esophagus characterized predominantly by eosinophilic inflammation, leading to esophageal dysfunction. Converging data have placed the esophageal epithelium at the center of disease pathogenesis. In particular, the main EoE disease susceptibility loci at 2p23 and 5p22 encode for gene products that are produced by the esophageal epithelium: the intracellular protease calpain 14 and thymic stromal lymphopoietin, respectively. Furthermore, genetic and functional data establish a primary role for impaired epithelial barrier function in disease susceptibility and pathoetiology. Additionally, the EoE transcriptome, a set of genes dysregulated in the esophagi of patients with EoE, is enriched in genes that encode for proteins involved in esophageal epithelial cell differentiation. This transcriptome has a high proportion of esophagus-specific epithelial genes that are notable for the unexpected enrichment in genes encoding for proteases and protease inhibitors, as well as in IL-1 family genes, demonstrating a previously unappreciated role for innate immunity responses in the esophagus under homeostatic conditions. Among these pathways, basal production of the serine protease inhibitor, Kazal-type 7 (SPINK7) has been demonstrated to be part of the normal differentiation program of esophageal epithelium. **Profound lost expression of *SPINK7* occurs in patients with EoE and is sufficient for unleashing increased proteolytic activity**

(including urokinase plasminogen activator), impaired barrier function, and production of large quantities of proinflammatory and proallergic cytokines, including thymic stromal lymphopoietin. Collectively, we put forth a model in which the esophagus is normally equipped as an anti-inflammatory sensing organ and that defects in this pathway, mediated by epithelial protease/protease inhibitor imbalances, unleash inflammatory responses resulting in disorders, such as EoE. (*J Allergy Clin Immunol* 2018;142:10-23.)

Key words: Eosinophilic esophagitis, eosinophilic oesophagitis, esophageal epithelium, epithelial differentiation, epithelial barrier, proteases, protease inhibitors, serine protease inhibitors, Kazal type

Eosinophilic esophagitis (EoE) is a chronic, allergen-driven eosinophilic inflammatory disease of the esophagus. The disease often coexists with other atopic disorders; has a growing prevalence reaching nearly 1:2000 subjects, indicating that it is considered a rare orphan disease; and is associated with an annual cost of \$1 billion in the United States alone.^{1,2} Symptoms include failure to thrive, vomiting, chest and abdominal pain, dysphagia, and food impaction, progressing in this order from childhood to adulthood.³ Compared with other chronic diseases of childhood, EoE has one of the lowest qualities of life, likely because of the restricted diets, chronic pain, relapsing nature, and need for recurrent endoscopies.^{4,5}

The pathogenesis of EoE involves a complex interplay of genetic, environmental, and immunologic components.⁶⁻⁸ Prior studies have demonstrated a high prevalence of white patients with EoE, and approximately 70% of the patients are male.⁹ A genetic component is supported by twin studies⁶ because monozygotic twins have 41% disease concordance compared with 22% in dizygotic twins and 2.4% in nontwin siblings of patients with EoE. These results highlight a critical contribution of genetics, as well as early-life environmental factors that account for more than 80% of the phenotypic variation of the disease.^{7,10-12} Perinatal risk factors for EoE include maternal or newborn fever, antibiotic use, proton pump inhibitor use, and admission into the neonatal intensive care unit. Interestingly, exposure to furry pets in the first year of life protects against EoE.¹³ Evidence is also emerging that implicates microbiota as a potentially critical factor in esophageal development, epithelial barrier function, and induction of EoE.¹⁴⁻¹⁶

EoE is considered to be a type 2 immune disease that often co-occurs with atopic diseases, including atopic dermatitis (AD), asthma, and food allergies.¹⁷⁻²⁰ As such, EoE is characterized by increased levels of the type 2 cytokines, which are critical for promoting cellular responses in patients with EoE. Early mouse studies revealed IL-5 as an important contributing factor for eosinophilic development and tissue infiltration and for remodeling.^{21,22} Similarly, IL-13 is highly upregulated in

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Terms in boldface and italics are detailed in the glossary on page 12.

Abbreviations used

AD:	Atopic dermatitis
ALI:	Air-liquid interface
BMP:	Bone morphogenetic protein
CAPN14:	Calpain 14
CST:	Cystatin
DSC:	Desmocollin
DSG1:	Desmoglein 1
DSP:	Desmoplakin
EDC:	Epidermal differentiation complex
EMSY (C11ORF30):	Chromosome 11 open reading frame 30
EMT:	Epithelial-mesenchymal transition
EoE:	Eosinophilic esophagitis
FA:	Food allergy
FLG:	Filaggrin
FST:	Follistatin
GWAS:	Genome-wide association study
IBF:	Impaired barrier function
IBL:	Interpapillary basal layer
IVL:	Involucrin
KLK:	Kallikrein
KRT:	Keratin
LRRC32:	Leucine-rich repeat-containing 32
NKX2.1:	NK2 homeobox 1
PAR2:	Protease-activated receptor 2
PBL:	Papillary basal layer
SERPIN:	Serine protease inhibitor
SNP:	Single nucleotide polymorphism
SOX2:	SRY-box-2
SPINK:	Serine protease inhibitor, Kazal type
SPRR:	Small proline-rich repeat
STAT:	Signal transducer and activator of transcription
SYNPO:	Synaptopodin
TAC:	Transit-amplifying cell
TGM:	Transglutaminase
TSLP:	Thymic stromal lymphopoietin
uPA:	Urokinase plasminogen activator
uPAR:	Urokinase plasminogen activator receptor
WES:	Whole-exome sequencing

esophageal biopsy specimens from patients with EoE,²³ and IL-13 overexpression in mice is sufficient to induce esophageal eosinophilia and other structural esophageal changes that are reminiscent of those in patients with EoE.²⁴ In addition, IL-13 stimulation in esophageal epithelial cells induces a molecular transcript signature that overlaps with the esophageal genes that are altered in patients with EoE, known as the EoE transcriptome.^{23,25} The critical role of IL-13 signaling in EoE pathogenesis is further supported by successful clinical trials of anti-IL-13 antibodies for the treatment of EoE.²⁶

Analysis of gene expression in human tissues revealed that the esophageal mucosa has a unique transcription signature of approximately 250 esophagus-specific genes (<https://www.proteinatlas.org/humanproteome/esophagus>).²⁷ Esophageal genes are considered to be enriched in the esophagus if their expression is at least 5-fold greater in the esophagus than in any other interrogated tissue. Nearly 40% of the esophagus-specific genes have dysregulated expression in patients with EoE, designated as Eso-EoE genes, with the majority being downregulated.²⁸ Strikingly, Eso-EoE genes were highly enriched in protease-related activities, uncovering proteolytic activity as an intrinsic property of the esophageal tissue and a putative

operational pathway in EoE pathogenesis. These genes included serine peptidase inhibitors from the serpin family (serine protease inhibitors [*SERPINS*]); serine protease inhibitors, Kazal-type (*SPINKs*); and the calpain protease calpain 14 (CAPN14), the gene for which is located in the 2p23 locus that is strongly genetically linked to increased EoE risk.^{29,30} Additionally, whole-exome sequencing (WES) analysis identified potentially damaging rare mutations in 18 Eso-EoE genes in patients with active EoE, some carrying more than 1 mutation.^{28,31} Collectively, these findings uncover potentially critical contributions of the esophageal epithelial responses to maintaining the epithelial barrier in the homeostatic esophagus and to the initiation and propagation of EoE.

Several recent reviews focused on EoE genetics and pathophysiology and involvement of multiple cells, including T and B cells, mast cells, basophils, eosinophils, and dendritic cells, in disease pathogenesis.³²⁻³⁵ In this review we will explore our understanding of the esophageal epithelium in the pathogenesis of EoE, focusing on epithelial genes, inflammatory pathways, barrier function, and the balance of proteases and protease inhibitors.

PROPERTIES OF THE ESOPHAGEAL EPITHELIUM

The esophagus is classically considered a simple organ involved in transferring food and liquids from the oral cavity to the stomach. The embryonic foregut gives rise to the esophagus, as well as the respiratory tract, stomach, liver, and pancreas. After expression of the transcription factors *NK2 homeobox 1* (*NKX2.1*) and *SRY-box-2* (*SOX2*), the anterior region of the foregut is differentiated into the trachea and esophagus, respectively.³⁶ Bone morphogenetic protein (*BMP*) and the *WNT signaling pathway* have been implicated in tracheal-esophageal separation. High levels of WNT signaling molecules preferentially induce NKX2.1 expression and thereby tracheal development, whereas BMP inhibitory molecules maintain high expression of SOX2, leading to esophageal epithelial stratification.³⁷⁻³⁹ Expression of the transcriptional factors SOX2 and *p63* is critical for proper esophageal epithelial stratification during development and maintaining esophageal homeostasis.³⁶ Notably, in the developed esophagus, inhibition of BMP signaling is required to keep basal layer progenitors at an undifferentiated stage, and increased expression of the BMP antagonist follistatin (FST) leads to hyperproliferation of the esophageal epithelium in patients with EoE.^{40,41}

The mucosa of the developed human esophagus is lined by the multilayer squamous nonkeratinized epithelium, which serves as a protective barrier against environmental insults, such as microorganisms, foods, and acid exposure.⁴² Histologically, the esophageal epithelium can be divided into 2 morphologically distinct regions: (1) the basal zone, with undifferentiated and proliferating cells, and (2) the suprabasal zone, consisting of progressively flattened cells with an increased degree of differentiation as they move closer to the lumen. The basal zone comprises the basal layer of cells in direct contact with the lamina propria and a few layers of dividing cells above the basal layer, which were defined as transit-amplifying cells (TACs). Notably and unlike the human esophagus, TACs in the mouse esophagus are localized to the basal layer of the esophageal epithelium.^{43,44} Basal layer cells can either overlay the epithelial papillae of the esophagus to form the papillary basal layer (PBL) or cover the

interpapillary zone to form the interpapillary basal layer (IBL; Fig 1).⁴⁵⁻⁴⁷ Cells at the basal layers include epithelial progenitors (stem cells), the main function of which is to maintain and renew esophageal epithelium in the homeostatic and damaged esophagus.⁴⁸ Notably, IBL cells divide asymmetrically, giving rise to 1 basal and 1 suprabasal cell, whereas PBL cells divide symmetrically, resulting in 2 basal cells.⁴⁶ This raises the possibility that IBL cells, but not PBL cells, are true esophageal epithelial progenitors. Despite uncertainty on the exact nature of the human esophageal progenitor population, progenitor properties of the basal layer cells are supported by their ability to form colonies that express markers of undifferentiated epithelium in culture and differentiate into esophageal spheroids in defined medium.^{49,50} Most superficial cells in the suprabasal zone of the epithelium are nondividing and considered terminally differentiated cells.

Consistent with their differentiation status and functional roles, esophageal epithelial cells in the basal and suprabasal zones retain distinct molecular characteristics. IBL cells remain largely nondividing, as defined by the lack of the proliferation markers Ki-67, phospho-histone H3, and proliferating cell nuclear antigen. The majority of the proliferation activity in the basal zone is observed in the epibasal layers of TACs, which stain positive for proliferation markers (Fig 1).^{45,46,51} Notably, IBL cells remain undivided, even in the inflamed esophagi of patients with EoE, whereas the TAC layer expands in patients with EoE, which is consistent with hyperproliferation of the basal zone of the epithelium.⁴⁷ Expression of the transcription factors p63, SOX2, integrins $\alpha 6$ and $\beta 1$, a low-affinity receptor for nerve growth factor

(p75 receptor), and keratin 5 (KRT5) and KRT14 is a hallmark of undifferentiated epithelial cells in the basal zone. Suprabasal zone cells primarily express more differentiated KRT4 and KRT13 and genes from the *epidermal differentiation complex (EDC)*.^{28,50,52-55} Both basal and suprabasal layers express E-cadherin in line with the epithelial origin of these cells.⁴⁷

Several findings suggest that basal layer cells can contribute uniquely to EoE pathogenesis. For example, in patients with EoE, IBL cells, but not other epithelial cells, markedly overexpress the innate epithelial cytokine IL-33, a prominent inducer of type 2 immune responses.^{47,56} In addition, expression of the actin-related protein synaptopodin (SYNPO), which is involved in regulating the esophageal epithelial barrier, is limited to basal layer cells in the homeostatic esophagus, whereas its expression expands into the suprabasal zone in patients with EoE.⁵⁷ Consistent with the role of BMP signaling in squamous epithelial differentiation, the BMP inhibitor FST is expressed in esophageal progenitors, and increased expression of FST in the suprabasal cells is linked to hyperproliferation.⁴¹ Taken together, these findings demonstrate a previously underappreciated complexity of esophageal epithelial responses during homeostasis and EoE.

Although the majority of esophageal genes are shared between the esophagus and other organs, a set of approximately 250 genes represents an expression signature of the esophageal mucosa and has been defined as esophageal identity genes.²⁸ These genes include a variety of structural proteins (ie, *KRT6C*, *KRT32*, involucrin [*IVL*], and desmoplakin [*DSP*]), small proline-rich repeats (SPRRs), enzymes (ie, transglutaminase 1 [*TGM1*] and *TGM3*), and a number of proteases and protease inhibitors (ie, SERPINS,

GLOSSARY

BONE MORPHOGENETIC PROTEINS (BMPs): A group of multifunctional growth factors belonging to the TGF- β superfamily. BMP inhibitory molecules maintain high SOX2 expression, leading to esophageal epithelial differentiation.

DESMOSOME: A hyperadhesive intercellular junction between individual cells that is crucial to epithelial barrier formation. Mutations in the desmosomal proteins desmocollin and desmoglein are linked to EoE pathogenesis and impaired barrier function.

EPIDERMAL DIFFERENTIATION COMPLEX (EDC): A gene locus comprising more than 50 genes (including filaggrin [*FLG*]) that encode for proteins involved in the terminal differentiation and cornification of squamous epithelial cells located at 1q21. Genes from this complex, including involucrin (*IVL*) and SPRR family members, are downregulated in patients with EoE and after IL-13 stimulation in esophageal epithelial cells in culture, leading to impaired barrier function, a primary feature of EoE pathogenesis. Mutations in *FLG* are genetically linked to atopic dermatitis and EoE.

EPITHELIAL-MESENCHYMAL TRANSITION (EMT): A process by which epithelial cells lose their cell polarity and cell-cell contacts and gain migratory and invasive properties. In patients with EoE, EMT is correlated with disease activity and likely contributes to subepithelial fibrosis.

IL-1 FAMILY: IL-1 family genes encode for up to 11 cytokines that bind to IL-1 family receptors, leading to initiation and regulation of the inflammatory response. Some cytokines and their receptors, including IL-36 α , IL-36RN, IL-1 α , and IL-1RN, are highly enriched in the esophagus compared with other tissues, and their expression is downregulated in patients with EoE. Conversely, expression of IL-33 is increased in patients with EoE in the subpopulation of esophageal epithelial progenitors in the basal layer of the epithelium.

NK2 HOMEBOX 1 (NKX2.1) AND SRY-BOX 2 (SOX2): Transcription factors involved in embryonic development of the trachea and esophagus, respectively.

p63: A member of the p53 gene family that codes for 6 known transcripts with transactivation or oncogenic effects on p53 reporter genes. It is a marker of epithelial progenitor cells.

SERPIN: A superfamily of proteins with similar structure that primarily function as irreversible serine protease inhibitors. Genetic variants in SERPINS are associated with food allergy.

SPINK: A family of serine peptidase inhibitors that contain inhibitory Kazal domains. SPINK proteins are expressed in various tissues and are usually secreted from the cells. SPINKs regulate the activity of extracellular serine proteases, and their dysregulation can promote epithelial barrier impairment and an innate immune response. SPINK5 deficiency causes Netherton syndrome with severe skin barrier impairment and atopy. SPINK7 is expressed in the esophageal epithelium, and its deficiency is associated with EoE pathogenesis.

UROKINASE PLASMINOGEN ACTIVATOR (uPA): Known as plasminogen activator, uPA is a serine protease involved in degradation of the extracellular matrix, thrombolysis, and eosinophil activation through its receptor, uPAR. Its gene is *PLAU*.

WNT SIGNALING PATHWAY: A group of evolutionarily conserved signal transduction pathways consisting of a variety of proteins that signal through cell-surface receptors that regulate organogenesis during embryogenesis. High levels of WNT signaling molecules preferentially induce NKX2.1 expression and thereby tracheal development.

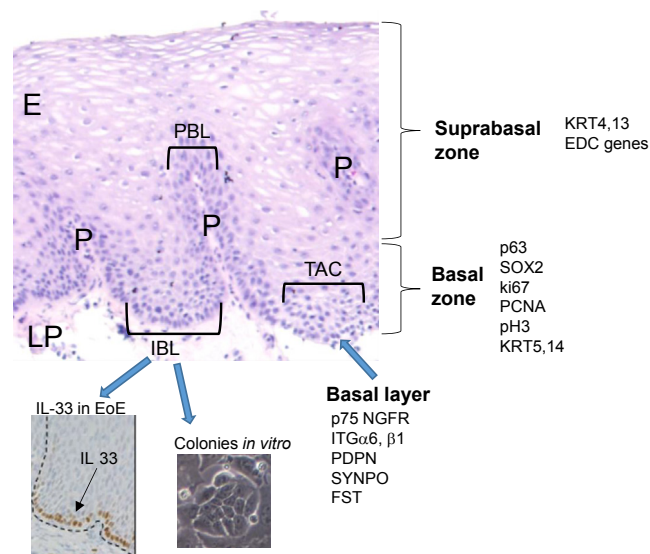


FIG 1. Basic organization of human esophageal epithelium. Nonkeratinized, stratified squamous epithelium (*E*) and the underlying lamina propria (*LP*) are shown. Invaginations of the epithelial layer form characteristic papillae (*P*). The epithelium is divided into the basal zone, which contains the IBL and PBL, several layers of dividing TACs, and the suprabasal zones of nondividing more differentiated cells. Prototypic markers of each cell layer of the epithelium are shown. Notably, IBL cells express IL-33 in patients with active EoE⁴⁷ and form colonies when grown *in vitro*. Part of the figure was extracted from Travers et al⁴⁷ with copyright permission.

SPINKs, kallikreins [KLKs], and *CAPN14*). Accordingly, functional enrichment analysis of the esophageal transcriptome shows enrichment in the epidermal development and differentiation, *desmosome* organization and barrier function, and serine-type and endopeptidase regulatory activity (Fig 2 and Table I).⁵⁸ The emerging contribution of esophageal proteases and protease inhibitors to EoE pathogenesis will be discussed further in this review.

Unexpectedly, the esophageal transcriptome contains the IL-1 receptor family of genes, including those of the cytokines IL-1 α , IL-36 α , and IL-36 β and their receptor antagonists IL-1RN and IL-36RN. Thus molecular functions related to IL-1 receptor binding is highly enriched in the homeostatic esophagus, as well as in patients with EoE, with decreased expression of IL-1 genes being observed in the Eso-EoE gene set and in esophageal epithelial cells in response to IL-13 (Fig 2 and Table I).²⁸ Moreover, expression of the *IL-1 family* member IL-33, which is a proallergic cytokine, is increased in IBL cells in patients with EoE, and its expression is normalized after successful treatment.⁴⁷ IL-36 cytokines are expressed predominantly by epithelial and immune cells and are involved in directly polarizing helper T cells, particularly in skin inflammatory responses. By modulating innate and adaptive immune responses, these cytokines regulate inflammatory responses in various tissues, including the skin and lungs.⁵⁹⁻⁶¹ Activation of the IL-1 family of cytokines requires proteolytic processing, likely by some of the enzymes that are normally expressed by differentiated esophageal epithelium. Although the exact role of IL-1 family members in patients with EoE is not known, we speculate that they are involved in the balancing of proinflammatory and anti-inflammatory responses.^{59,62} Collectively, these findings unveil previously underappreciated innate immune properties of the esophageal epithelium and highlight esophagus-specific genes as possible regulators of esophageal homeostasis and that their

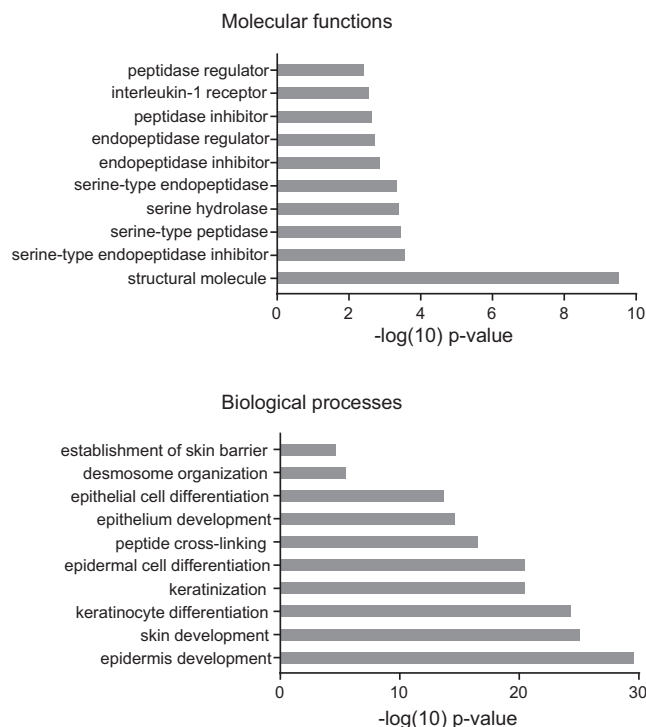


FIG 2. Functional enrichment analysis of the esophageal transcriptome. The top 10 overrepresented gene ontology terms for molecular functions and biological processes for the esophagus-specific genes obtained from the Human Protein Atlas are shown. Genes associated with enriched pathways are shown in Table I. Enrichment analysis was performed with ToppFun (<https://toppgene.cchmc.org/enrichment.jsp>).⁵⁸

aberrant expression possibly contributes to the pathogenesis of EoE.

LOSS OF ESOPHAGEAL TISSUE DIFFERENTIATION IN PATIENTS WITH EoE

EoE is characterized by a unique esophageal gene expression signature designated as the EoE transcriptome.^{23,25} Initially, the EoE transcriptome was assessed by using expression array analysis and included 574 genes, which distinguished patients with EoE from healthy control subjects and from patients with gastroesophageal reflux disease.²³ Later, this transcriptome was refined by using RNA sequencing of esophageal biopsy specimens from healthy control subjects and patients with active EoE, identifying a total of 1607 significantly dysregulated transcripts with around two thirds upregulated and one third downregulated genes.²⁵ A considerable fraction (15%) of the genes expressed in homeostatic esophagus by using RNA sequencing overlapped with genes dysregulated in *in vitro*-differentiated esophageal epithelial cells grown in air-liquid interface (ALI) conditions.⁶³ Moreover, IL-13 stimulation of these cells induced gene signature and disease-associated pathways, which overlapped with those observed in the inflamed esophageal mucosa of patients with EoE.^{28,63} These data reinforce the role of IL-13 as a molecular driver of EoE and underscored a substantial contribution of the esophageal epithelial transcriptome to the overall extent of transcriptional responses in patients with EoE.

Although the EoE transcriptome represents the gene expression signature of a number of cell populations involved in EoE

TABLE I. Molecular functions and biological processes enriched in esophagus-specific genes*

Name	P value†	Hits in query list
Molecular functions		
Structural molecule activity	2.71E-10	<i>MYH8, MAL, SPRR1A, SPRR1B, SPRR2A, SPRR2B, SPRR2D, SPRR2E, SPRR2F, SPRR3, EVPL, IVL, JUP, KRT24, KRT6C, CLDN17, PKP1, KRT3, KRT4, KRT5, KRT6A, KRT6B, CRCT1, KRT13, KRT78, KRT15, KRT16, DSP, KRT31, KRT32, KRT33A, ANXA1, LAD1, PPL, LCE3E, EVPL, KRTAP3-2, CSTA</i>
Serine-type endopeptidase inhibitor activity	2.42E-04	<i>PI3, SERPINB5, SERPINB13, SPINK7, SERPINB3, SERPINB4, SERPINB11, A2ML1, SPINK5, SERPINB2</i>
Serine-type peptidase activity	3.13E-04	<i>KLK7, KLK6, KLK10, PRSS22, TMPRSS11F, TMPRSS11B, TMPRSS11D, ENDOU, KLK11, TMPRSS11E, KLK12, PRSS27, KLK8, KLK13, TMPRSS11A</i>
Serine hydrolase activity	3.66E-04	<i>KLK7, KLK6, KLK10, PRSS22, TMPRSS11F, TMPRSS11B, TMPRSS11D, ENDOU, KLK11, TMPRSS11E, KLK12, PRSS27, KLK8, KLK13, TMPRSS11A</i>
Serine-type endopeptidase activity	4.20E-04	<i>KLK7, KLK6, KLK10, PRSS22, TMPRSS11F, TMPRSS11B, TMPRSS11D, KLK11, TMPRSS11E, KLK12, PRSS27, KLK8, KLK13, TMPRSS11A</i>
Endopeptidase inhibitor activity	1.22E-03	<i>PI3, SERPINB5, SERPINB13, SPINK7, SERPINB3, SERPINB4, SERPINB11, A2ML1, SPINK5, SERPINB2, CSTA, CSTB</i>
Endopeptidase regulator activity	1.75E-03	<i>PI3, SERPINB5, SERPINB13, SPINK7, SERPINB3, SERPINB4, SERPINB11, A2ML1, SPINK5, SERPINB2, CSTA, CSTB</i>
Peptidase inhibitor activity	2.09E-03	<i>PI3, SERPINB5, SERPINB13, SPINK7, SERPINB3, SERPINB4, SERPINB11, A2ML1, SPINK5, SERPINB2, CSTA, CSTB</i>
IL-1 receptor binding	2.60E-03	<i>IL36B, IL36A, IL36RN, IL1A, IL1RN</i>
Peptidase regulator activity	3.41E-03	<i>MAL, PI3, SERPINB5, SERPINB13, SPINK7, SERPINB3, SERPINB4, SERPINB11, A2ML1, SPINK5, SERPINB2, CSTA, CSTB</i>
Biological processes		
Epidermis development	1.94E-30	<i>KLK7, CNFN, SPRR1A, SPRR1B, SPRR2A, SPRR2B, SPRR2D, SPRR2E, SPRR2F, SPRR3, EVPL, SCCL, TGM5, IVL, GRHL1, GJB5, SFN, SPINK5, FOXN1, KRT5, KRT6B, CRCT1, KRT15, KRT16, DSP, KRT31, KRT32, ANXA1, TMEM79, BARX2, CRABP2, PPL, LCE3E, ZNF750, TGM1, TGM3, OVOL1, SOX21, EVPL, EPHA2, TP63, CERS3, CSTA, GRHL3, IL1A, DKK4</i>
Skin development	6.65E-26	<i>CNFN, SPRR1A, SPRR1B, SPRR2A, SPRR2B, SPRR2D, SPRR2E, SPRR2F, SPRR3, EVPL, SCCL, IVL, JUP, GRHL1, GJB3, ALOX12, SFN, SPINK5, FOXN1, KRT6B, CRCT1, KRT16, DSP, ANXA1, TMEM79, BARX2, PPL, LCE3E, TGM1, TGM3, OVOL1, SOX21, EPHA2, TP63, CERS3, CSTA, GRHL3, IL1A, DKK4</i>
Keratinocyte differentiation	3.64E-25	<i>CNFN, SPRR1A, SPRR1B, SPRR2A, SPRR2B, SPRR2D, SPRR2E, SPRR2F, SPRR3, EVPL, SCCL, IVL, GRHL1, SFN, FOXN1, KRT6B, CRCT1, KRT16, DSP, ANXA1, TMEM79, PPL, LCE3E, TGM1, TGM3, EPHA2, TP63, CERS3, CSTA, IL1A</i>
Keratinization	2.21E-21	<i>CNFN, SPRR1A, SPRR1B, SPRR2A, SPRR2B, SPRR2D, SPRR2E, SPRR2F, SPRR3, EVPL, IVL, SFN, KRT6B, KRT16, TMEM79, PPL, LCE3E, TGM1, TGM3, IL1A</i>
Epidermal cell differentiation	2.40E-21	<i>CNFN, SPRR1A, SPRR1B, SPRR2A, SPRR2B, SPRR2D, SPRR2E, SPRR2F, SPRR3, EVPL, SCCL, IVL, GRHL1, SFN, SPINK5, FOXN1, KRT6B, CRCT1, KRT16, DSP, ANXA1, TMEM79, PPL, LCE3E, TGM1, TGM3, EPHA2, TP63, CERS3, CSTA, IL1A</i>
Peptide cross-linking	2.29E-17	<i>SPRR1A, SPRR1B, SPRR2A, SPRR2B, SPRR2D, SPRR2E, SPRR2F, SPRR3, EVPL, TGM5, IVL, CRCT1, DSP, ANXA1, LCE3E, TGM1, TGM3, CSTA</i>
Epithelial development	1.73E-15	<i>KLK7, CNFN, SPRR1A, SPRR1B, SPRR2A, SPRR2B, SPRR2D, SPRR2E, SPRR2F, SPRR3, EVPL, SCCL, AKR1C2, TGM5, PTK6, PRDM1, IVL, GRHL1, SERPINB5, GJB5, NKX6-1, SDC1, ALOX12, SFN, SPINK5, RHCG, FOXN1, KRT3, KRT4, KRT5, KRT6B, CRCT1, RAB25, KRT15, KRT16, UPK1A, DSP, KRT31, KRT32, ANXA1, TMEM79, RIPK4, BARX2, CRABP2, PPL, LCE3E, ZNF750, TGM1, TGM3, OVOL1, SOX21, EVPL, EPHA2, TP63, CERS3, CSTA, GRHL3, IL1A, TACSTD2, DKK4</i>
Epithelial cell differentiation	1.36E-14	<i>CNFN, SPRR1A, SPRR1B, SPRR2A, SPRR2B, SPRR2D, SPRR2E, SPRR2F, SPRR3, EVPL, SCCL, AKR1C2, PTK6, PRDM1, IVL, GRHL1, NKX6-1, SDC1, ALOX12, SFN, SPINK5, RHCG, FOXN1, KRT3, KRT4, KRT6B, CRCT1, RAB25, KRT16, UPK1A, DSP, ANXA1, TMEM79, PPL, LCE3E, TGM1, TGM3, EPHA2, TP63, CERS3, CSTA, IL1A</i>
Desmosome organization	2.30E-06	<i>PERP, JUP, GRHL1, NECTIN1, DSP, PKP3</i>
Establishment of skin barrier	1.42E-05	<i>GRHL1, ALOX12, SFN, KRT16, TMEM79, TP63, GRHL3</i>

*Enrichment analysis was performed with TopPFun (<https://toppgene.cchmc.org/enrichment.jsp>).

†Adjusted P value, Bonferroni correction.

pathogenesis (ie, mast cells and eosinophils), the majority of the transcriptional changes are driven by epithelial responses.^{64,65} Accordingly, the EoE transcriptome revealed a significant down-regulation of multiple structural genes, including filaggrin (*FLG*),

IVL, and the *SPRR* gene family located at chromosome region 1q21, which constitutes the EDC. Changes in genes located at 1q21 occur more frequently than any other loci in the genome.⁵⁵ Given that the expression of some of these structural genes is

highly enriched in the esophagus compared with in other tissues,²⁸ these data suggest that tissue-specific transcriptional dysregulation drives the inflammatory response in patients with EoE. Indeed, intersection of the EoE transcriptome with esophagus-specific genes revealed that around 40% of the esophageal genes were altered transcriptionally and that nearly 90% of these genes, designated as Eso-EoE genes, were downregulated in patients with EoE.²⁸ The Eso-EoE genes were enriched for genes involved in pathways related to keratinization, epidermal development, and differentiation.²⁸

Accordingly, impaired barrier function (IBF) is a hallmark of allergic inflammation in patients with EoE, which has been previously linked to decreased expression of the desmosomal protein desmoglein 1 (DSG1)^{66,67} and loss of expression of structural epithelial genes from the EDC after epithelial response to IL-13, as well as imbalanced expression of proteases and their inhibitors.^{28,55,68-70} Similar to alterations in the esophageal transcriptome, major dysregulation in protease-related activities and IL-1-related genes were revealed by means of functional enrichment analysis of Eso-EoE genes. Although *CAPN14* and *SERPINS* were highly upregulated, *SPINKs* were downregulated in patients with EoE. IL-1-related cytokines and receptors were also downregulated in patients with EoE. Notably, WES performed in 33 unrelated patients with EoE identified 39 rare variants (<1% minor allele frequency) that were located in 18 Eso-EoE genes, including the protease inhibitors *SERPINB3*, *SPINK5*, and alpha-2-macroglobulin like 1 (*A2ML1*); *CAPN14*; the structural proteins *KRT6B* and desmocollin 2 (*DSC2*); and a member of the peptidyl arginine deiminase family, peptidyl arginine deiminase 3 (*PADI3*).²⁸ Furthermore, rare variants in mitochondrial genes expressed in a variety of cells, including esophageal epithelium, are likely contributing to EoE pathogenesis and phenotype.³¹ Collectively, these findings provide potential mechanisms for genetically driven, tissue-specific alterations in patients with EoE and underscore the centrality of proteolytic activity for the disease pathogenesis.

EPITHELIAL BARRIER DYSFUNCTION IN PATIENTS WITH EoE

The healthy, multilayered esophageal epithelium constitutes a defensive mechanism against environmental factors, including food and acid refluxate, penetrating into esophageal tissue and invoking structural damages or inflammatory responses. Unlike the stomach and duodenum, the esophagus has no thick surface mucous layer, and unlike the mouse esophageal epithelium, human epithelium is not keratinized. Therefore the integrity of the esophageal epithelium provided by tight junctions, adherence junctions, and desmosomes is paramount to carrying out its protective function.⁷¹ Morphologically, inflamed esophageal epithelium in patients with EoE is characterized by hyperproliferation of the basal zone of the epithelium, which replaces most of the more differentiated upper layer of epithelial cells and the appearance of dilated intercellular spaces.^{66,72} Importantly, these changes are reproduced in culture in esophageal epithelium differentiated in the presence of IL-13 and are accompanied by loss of barrier function, as measured by using transepithelial electrical resistance and increased permeability.⁶³ Collectively, these changes constitute IBF, which is a hallmark of allergic inflammation in patients with EoE.

On the molecular level, IBF is linked to dysregulation of structural genes that comprise tight junctions (claudins 1 and 7

and occludin),^{69,73,74} adherens junctions (E-cadherin), and desmosomes (*DSG1*),^{66,69} leading to decreased numbers of desmosomes in patients with EoE.⁷²

Accordingly, in patients with EoE, as well as in primary esophageal epithelial cells, the esophageal epithelium exhibits characteristics of *epithelial-mesenchymal transition (EMT)*, as evidenced by increased expression of vimentin and α smooth muscle actin and decreased expression of E-cadherin.⁷⁵⁻⁷⁷ Altered epithelial differentiation is demonstrated further by lost expression of *FLG*, *IVL*, and several *SPRR* family members (*SPRR2C*, *SPRR2D*, and *SPRR3*).^{28,55,68} Notably, *FLG* is expressed on mucosal surfaces and is essential to intact barrier formation because it promotes aggregation of keratin-intermediate filaments into macrofibrils, which adds to the physical strength of the epithelium. *FLG* monomers are incorporated into the lipid envelope, where they undergo additional protease-dependent processing to release free amino acids that assist in water retention and sealing of the intercellular spaces. These processes block exogenous immune stimuli from entering the epithelium and activate immune responses.⁷⁸ *FLG* dysfunction has been associated previously with defects in epidermal barrier function in patients with AD, which frequently co-occurs with EoE.⁷⁹ In addition, mutations in *FLG*, as well as in the esophagus-specific genes *KRT6B* and *DSC2*, are identified in patients with EoE.^{28,55}

EDC genes are expressed in esophageal epithelial cells and are downregulated in patients with EoE and in response to IL-13 *in vitro*.⁵⁵ In contrast, the recently characterized cadherin-like 26, which was suggested to regulate barrier function, is highly upregulated in patients with EoE, as well as those with eosinophilic gastritis and in epithelial cells after IL-13 stimulation.⁸⁰ Another recent finding implicated the actin-related protein SYNPO, which is also highly upregulated in patients with EoE, in regulating esophageal barrier integrity. Forced expression of SYNPO in esophageal epithelial cells grown at the ALI resulted at the IBF accompanied by transcriptional changes in structural genes.⁵⁷

These collective data indicate that altered esophageal epithelial integrity is a critical contributor to allergic inflammation in patients with EoE.

GENETIC AND EPIGENETIC MECHANISMS IN EPITHELIAL RESPONSES IN PATIENTS WITH EoE

A central question in the allergy field is why allergic responses manifest in a tissue-specific manner, such as EoE. A number of studies used phenotypic and genetic association and candidate-gene and genome-wide association study (GWAS) approaches to identify multiple genetic variants associated with increased risk of EoE.^{29,30,55,64,81-83} Notably, most genes associated with increased susceptibility to EoE are expressed in the esophageal epithelium, substantiating the role of epithelial responses in EoE pathogenesis (Table II). Among these genes are proinflammatory epithelium-derived cytokines, including thymic stromal lymphopoietin (TSLP) and IL-33, as well as signal transducer and activator of transcription 6 (STAT6), a transcription factor activated by the type 2 cytokines IL-4 and IL-13, which drive the EoE transcriptome, at least in part.^{23,30,34} Notably, mutations in tumor suppressor lipid phosphatase and tensin homolog (*PTEN*) have been linked previously to hamartoma tumor syndrome, and the risk of this syndrome and *PTEN* mutations are increased in patients with EoE.⁸⁴

Recently, we reported that genetic susceptibility to EoE is mediated at least in part by synergistic interactions between single

TABLE II. Epithelium-derived proteins with genetic association with EoE

Protein name	Protein family	Putative function in patients with EoE
CAPN14*	Calpain protease	Regulates epithelial barrier homeostasis and repair
EMSY (C11ORF30)*	Nuclear protein	Possibly regulates transcription and chromatin remodeling
STAT6*	Transcription factor	IL-4/IL-13 signaling pathway
TSLP*	Common receptor γ chain cytokine	Induces allergic inflammation by activating immune cells
ANKRD27 (VARP)*	Possibly a guanine exchange factor	Regulates enzyme trafficking
FLG†	Filament-associated protein	Aggregates keratin intermediate filaments in differentiation
CCL26†	Chemokine	Eosinophilic chemoattractant
IL-33†	IL-1 family cytokine, alarmin	Induces allergic inflammation, might regulate gene expression
DSP/DSG1†‡	Structural proteins	Major components of the desmosome; regulate barrier integrity
SPINK5‡	Serine protease inhibitor, Kazal-type	Contributes to barrier integrity
PTEN‡	Phosphatase	Negative regulation of signaling pathways, might regulate eosinophilic responses

ANKRD27, Ankyrin repeat domain 27; C11ORF30, chromosome 11 open reading frame 30; DSG1, desmoglein; PTEN, phosphatase and tensin homolog; SPINK5, serine peptidase inhibitor, Kazal-type 5.

*Identified by means of GWAS.

†Candidate genes.

‡Mendelian genetic association.

nucleotide polymorphisms (SNPs) in the loci encoding for *TSLP* and *IL4*, which is consistent with the allergic cause of EoE and co-occurrence of EoE with asthma, allergic rhinitis, and AD.⁸⁵ Furthermore, mutations in genes implicated in maintaining barrier integrity in those atopic diseases, including *FLG* and *DSG1*, have been also linked with EoE.^{55,66,67,86,87} Interestingly, SNPs near the chromosome 11 open reading frame 30 (*EMSY* [*C11ORF30*]) gene at 11q13 are associated with EoE susceptibility. *EMSY* is expressed in multiple types, including the epithelium, and is implicated in the DNA damage response.^{88,89} It is increased in patients with EoE and is likely involved in regulating the expression of *TSLP* and *CCL5*.⁹⁰ Another epithelium-derived protein, ankyrin repeat domain 27 (VARP), which regulates membrane trafficking and endosome dynamics,^{91,92} has been implicated in the pathogenesis of EoE based on genetic linkage, but the mechanism is yet to be explored.

Genes linked with EoE share a significant proportion of the esophagus-specific genes recently reported by the Human Protein Atlas.²⁸ The strongest associated EoE risk locus is 2p23, which encodes for the esophagus-specific protease CAPN14.^{29,30} CAPN14 is a member of the classical calcium-activated calpain family, which is defined by its similar domain structure to the most extensively studied calpains, CAPN1 and CAPN2.⁹³ CAPN14 is dynamically upregulated as a function of EoE disease activity and after exposure of esophageal epithelial cells to IL-13. Calpains are involved in the number of biological processes related to processing of cytoskeletal and membrane proteins, signal transduction pathways, and enzymes controlling cell-cycle progression, gene expression, and apoptosis.⁹⁴ Overexpression of CAPN14 leads to IBF in the esophageal epithelial cells grown at ALI and is accompanied by loss of expression of DSG1, an intercellular junctional protein implicated in barrier formation.⁹⁵ Recently, a WES analysis of patients with EoE identified rare damaging mutations in *SPINK5* and *SERPINB3*,²⁸ suggesting critical involvement of esophageal proteases and protease inhibitors in the pathogenesis of EoE, likely by maintaining the epithelial barrier.

A fundamental question in understanding the pathogenesis of EoE is how genetic risk loci and the environment cross-talk to promote the disease. A recent case-control study assessed an interplay between genetic risk loci and early-life environmental factors, including breast-feeding and hospital admission.⁷ The

authors discovered that the EoE risk associated with harboring the *CAPN14* susceptibility genotype can be significantly decreased by breast-feeding, further supporting the contribution of the environment to occurrence of EoE. GWASs revealed that the vast majority of EoE risk variants are localized to intronic and intergenic regions and do not directly affect coding regions of the associated genes.^{29,34} The question then remains of how these variations in genetic code are translated into the phenotypic outcome of the disease. It is well documented that SNPs are often localized to gene regulatory elements, including promoters and enhancers.⁹⁶ Activity of these elements is modified by environmental cues, including cytokines, which elicit dynamic changes in histone acetylation and methylation and subsequently in gene expression.⁹⁷ Thus epigenetic profiling combined with gene expression analysis serves as a useful approach to discover molecular mechanisms behind genetic associations, especially because the prevalence of EoE is influenced by gene-environment interactions.⁶ Indeed, expression of several epithelial genes involved directly in the pathogenesis of EoE is under epigenetic regulation. These genes include *CCL26*, neurotrophic receptor tyrosine kinase 1 (*NTRK1*), *SYNPO*, and *CAPN14*.^{57,98-100} In the case of *CAPN14*, the EoE risk variant rs76562819 is localized in the peak of histone H3 lysine 27 acetylation, which is dynamically regulated by IL-13 stimulation. Importantly, *CAPN14* was expressed in a haplotype-dependent manner, with subjects with the risk allele expressing 30% less *CAPN14* than those without the risk haplotype.²⁹ Decreased expression of *CAPN14* was correlated with the ability of the risk allele to interact with an as yet to be identified nuclear protein; however, the effect of risk and non-risk alleles on the epigenetic status of the gene has not been assessed. Although how the molecular mechanisms related to these genetic variations contribute to phenotypic outcome is yet to be determined, differential expression of esophageal genes located within 25 kb of EoE risk loci was sufficient to segregate patients with EoE from the control subjects.²⁹ Collectively, these findings unify transcriptional and epigenetic responses of esophagus-specific genes and provide the framework for future studies of gene regulatory mechanisms in patients with EoE.

Given that EoE is triggered primarily by exposure to food allergens, genetic predisposition to food allergy (FA) provides additional insight into EoE pathogenesis. Unlike EoE, a previous GWAS linked FA with the human MHC region encoding for the

EoE	Common	FA
<i>CAPN14</i> * <i>ANKRD27</i>	<i>TSLP/WDR36</i> <i>STAT6</i> # <i>LRRC32/EMSY</i> <i>FLG</i> #	<i>HLA-DQ/DR</i> <i>SERPINB7</i> <i>SERPINB2</i>

FIG 3. Genetic associations in patients with EoE and those with FA. Genetic risk loci for EoE and FA were identified by using GWASs^{29,30,101-104} and candidate-gene studies.^{55,109,111} Genes associated with individual and common risk loci are shown. *Genes with damaging rare mutations identified in WES.²⁸ #Genes identified by using a candidate-gene approach (*STAT6* for patients with FA and *FLG* for patients with EoE).

HLA genes.¹⁰¹⁻¹⁰³ A recent GWAS identified 5 loci associated with FA.¹⁰⁴ Similar to EoE, FA risk variants were associated with the *FLG* locus, signifying the importance of barrier integrity in both pathologies.⁵⁵ Consistent with the role of TGF- β signaling in patients with FA and EoE^{74,105,106} and in agreement with a previous report, association was found with leucine-rich repeat-containing 32 (*LRRC32*).⁸³ Although the function of *LRRC32* in patients with EoE is not known, it is reported to be a TGF- β binding protein on the surfaces of regulatory T cells.^{107,108} Moreover, similar to EoE, FA was genetically linked to SERPINS, in the case of FA to *SERPINB7/B2*. Although the mechanism of how SERPINS contribute to FA is not known, both we and the original authors of the FA GWAS article speculate that this could be related to tissue-specific expression of these genes, perhaps in the esophagus.

Given the frequent co-occurrence of EoE and AD, a candidate-gene approach was used to examine genetic association between FA, EoE, and AD by using risk loci previously found with a GWAS.¹⁰⁹ The authors identified 14 genetic loci in significant association with FA, including the *STAT6* and *TSLP/WDR6* loci, which were common between patients with FA and those with EoE. Interestingly, genetic variants in the *TSLP* locus associate with increased numbers of food triggers in patients with EoE.¹¹⁰ The role of the *STAT6* gene variants in patients with FA is further supported by association of the specific SNPs in the *STAT6* gene with more severe symptoms during food challenges.¹¹¹ Similar to the GWAS analysis,¹⁰⁴ the candidate-gene analysis identified *FLG* as a common risk factor for AD and FA, again reinforcing the importance of barrier integrity.¹⁰⁹

Collectively, these data show significant functional overlap between patients with FA and those with EoE in several genes linked to the epithelial barrier, type 2 response, and protease activity, yet some associations, such as *CAPN14*, remain specific to EoE, emphasizing the role of esophageal epithelial genes in the pathophysiology of EoE (Fig 3).^{28-30,34,55,101-104,109,111}

PHYSIOLOGIC ROLE OF PROTEASES AND THEIR INHIBITORS IN EPITHELIAL CELL DIFFERENTIATION AND BARRIER FORMATION

Stratified esophageal epithelium comprises a few layers of epithelial cells that not only provide a barrier from the external environment but also sense and respond to perturbations to eliminate toxic stimuli. This sophisticated role of the epithelium is controlled by a series of ordered processes that occur during formation of the epithelial barrier and homeostasis. A distinct repertoire of proteases and inhibitors is found in each layer of the

epithelium and resides in various cellular compartments, as well as in the extracellular space.¹¹² Proteases catalyze the hydrolysis of peptide bonds on their target substrates in a fundamentally irreversible reaction¹¹³; therefore their activity is tightly regulated. An activation cascade, in which other proteases often remove a propeptide from the nonactive protease, is required for the protease to be activated. Once a protease is activated, a protease inhibitor can bind to it and inhibit the proteolytic activity. In addition, the microenvironment regulates the activity of the proteases by controlling the optimal pH and the abundance of ions, such as calcium and zinc, that can either stimulate or inhibit the proteolytic activity.^{114,115} The balance between proteases and their tailored protease inhibitors dictates the steps required for generation and maintenance of the epithelial barrier and serves as a mechanism that senses damages and environmental insults, allowing immune response and regeneration of damaged tissues.

Imbalance between proteases and their inhibitors is a hallmark of several disorders; the number of the known pathologic conditions caused by altered proteolytic activity increased dramatically over recent years and includes many diseases. For example, a highly investigated protease is **urokinase plasminogen activator (uPA)**, which cleaves and activates plasminogen into active plasmin. uPA binds to its urokinase plasminogen activator receptor (uPAR) on the leading migratory edge of tumor cells and enhances tumor cell migration and invasion by degrading extracellular matrix components, thereby promoting metastatic development of several cancers. In addition, proteases drive cell-cycle progression by regulating the activity of cyclin-dependent kinases. Therefore dysregulation of proteases leads to altered proliferation.^{116,117}

Another example comes from Alzheimer disease, in which amyloid β -peptide levels in the brain are the primary trigger for neuronal cell death. Levels of amyloid β -peptide are dictated by the balance between its synthesis and removal. Currently, more than 20 amyloid-degrading proteases are implicated in the process of amyloid peptide processing and degradation, and several of those are decreased in specific brain areas with age or after hypoxia, ischemia, or stroke, thus contributing significantly to the development of Alzheimer disease pathology.^{113,117,118} Therefore dysregulation of proteases is an emerging key mechanism in the pathogenesis of multiple pathologies.

In the stratified epithelium a tight epithelial barrier is progressively formed after basal epithelial cell proliferation, movement of the cells outward toward the epithelial surface, initiation of the differentiation program, and subsequent formation of intercellular junctional complexes. Epithelial fate is regulated by membrane-bound a disintegrins and metalloproteases, which expose and process ligands and receptors (eg, members of the Delta/Serrate/lag-2 and Notch families) to the keratinocytes in the skin, switching from proliferation to differentiation mode.¹¹⁹ In the differentiated epithelial layers of the skin, intracellular organelles are destroyed after TGM-mediated cross-linking and assembling multiple proteins and cornified envelope components. This process is regulated by a protease cascade and protease inhibitors.^{120,121} Subsequently, proteases promote final maturation of connective tissue elements, such as FLG.¹¹² After a proper barrier is achieved, the desquamation process occurs. In this process, corneodesmosomal components, such as corneodesmosin, DSG1, and DSC1, are cleaved, and cells are detached from the tissue. Cathepsin D, KLK5, and KLK7 cleave these corneodesmosomal proteins

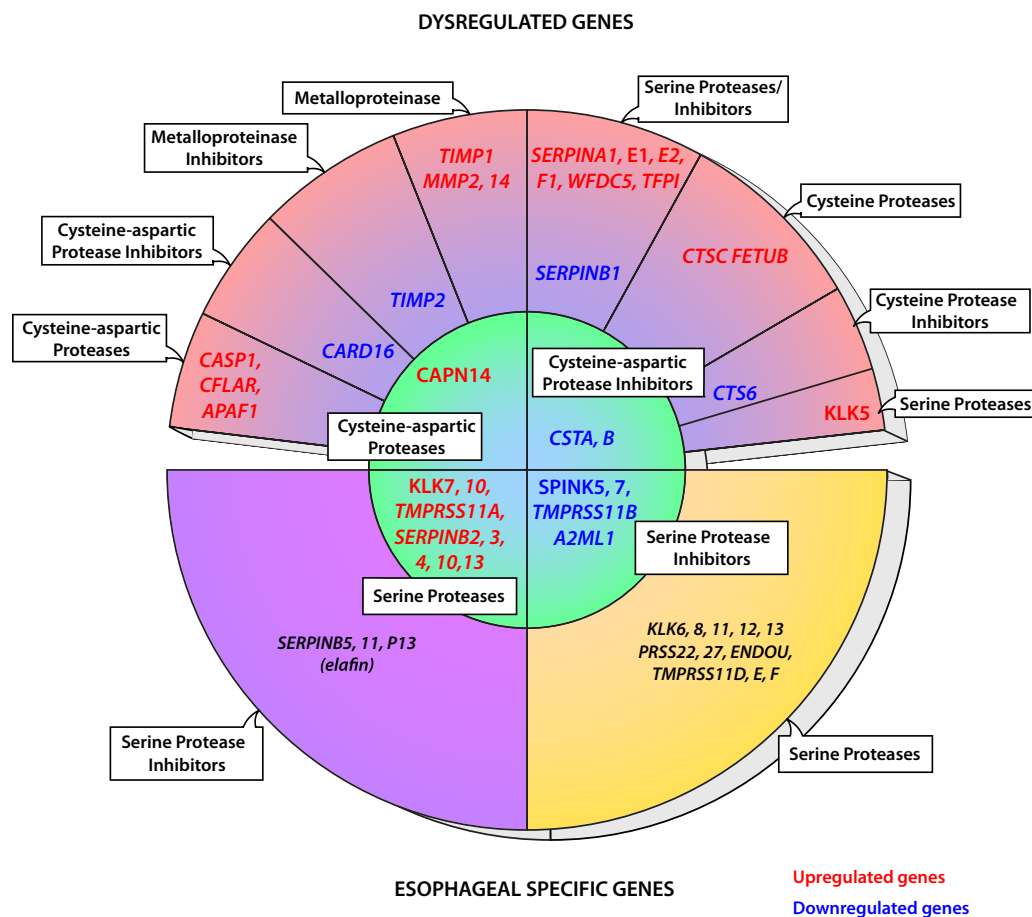


FIG 4. Summary of proteases and protease inhibitors with putative roles in EoE pathogenesis grouped according to their origins. Esophageal enriched protease and protease inhibitors were identified by using proteomic analysis.²⁷ Proteases and protease inhibitors with altered expression in patients with EoE were identified by using RNA sequencing.⁶⁶ Genes in italics indicate genes with altered mRNA expression in patients with EoE, and genes not in italics indicate altered protein expression in patients with EoE. The inner green circle represents esophageal specific genes that are dysregulated in EoE. Classification of the genes in the inner green circle are indicated in the rectangular boxes.

during homeostasis.^{122,123} Notably, IBF in patients with EoE is linked to the loss of epithelial DSG1, likely mediated in part by increased expression of CAPN14.^{66,95}

Unlike in the skin, the role of KLKs in the esophagus is much less investigated. Analysis of esophagus-specific genes in public data sets reveals that several KLKs (ie, KLK5, KLK7, KLK8, KLK10, KLK11, KLK12, and KLK13) are esophagus-enriched genes. Expression of KLK7 and KLK10 is increased in patients with active EoE compared with that in control subjects (Fig 4),¹²⁴ suggesting that KLKs might regulate esophageal epithelial barrier maintenance and that altered KLK expression and/or activity might be an inducing factor in barrier impairment. Lastly, regeneration of the tissue after damage is mostly orchestrated by the balance between the extracellular matrix-degrading proteases (ie, metalloproteases) and tissue inhibitors of metalloproteases.¹²⁵ These processes are likely involved in EMT, which is found in patients with EoE.⁷⁵

ALTERATIONS IN PROTEASES AND PROTEASE INHIBITORS IN PATIENTS WITH EoE

Recent publications highlighted the role of the cysteine protease CAPN14 in EoE pathology. CAPN14 has a unique expression

pattern, with the highest levels in the upper gastrointestinal tract, particularly in the squamous epithelium of the esophagus. CAPN14 expression is induced in the esophagi of patients with EoE compared with control subjects. In addition, CAPN14 is the only calpain that is upregulated by IL-13. Overexpression of CAPN14 in esophageal epithelial cells is sufficient to induce disruption of epithelial cell architecture and IBF, probably by downregulating DSG1, at least in part. Downregulation of CAPN14 disturbs IL-13-induced epithelial cell changes, as demonstrated by increased dilated intercellular spaces and basal cell disorder after CAPN14 gene silencing.⁹⁵ Interestingly, 2 cysteine inhibitors, cystatin (CST) A and CSTB, are highly enriched in the esophagus, and their expression is significantly downregulated in patients with EoE (Fig 4).^{27,66} Notably, loss-of-function mutations in CSTA results in acral peeling skin syndrome.¹²⁶ Although inhibitory activity of CST toward CAPN14 has not been shown, it is intriguing to speculate that CSTs can modulate CAPN14 activity in patients with EoE. Given the important emerging role of CAPN14 in patients with EoE, identifying CAPN14 substrates and elucidating the natural, tissue-resident inhibitors of CAPN14 will be beneficial for elucidating the molecular mechanisms that are altered in patients with EoE and for targeting this pathway for therapeutic intervention.

Along with dysregulated expression of proteases, expression of several protease inhibitors is also altered in patients with EoE, indicating putative proteolytic imbalance. For example, several lines of evidence suggest a role for the protease inhibitor lymphoepithelial Kazal-type-related inhibitor (also known as SPINK5) in EoE pathoetiology. The role of SPINK5 was investigated primarily in the development of barrier function and type 2 immune response in the skin. Loss-of-function mutation of *SPINK5* results in a rare disease termed Netherton syndrome, which is characterized by a severe hyperatopy phenotype mainly involving loss of the skin barrier, development of atopic manifestations with increased IgE levels, and recurrent infections (likely because of loss of the skin barrier).¹²⁷ Imbalance between SPINK5 and KLK proteases has been proposed to contribute to disease pathogenesis.¹²⁸ High prevalence of esophageal eosinophilia was observed in a cohort of patients with Netherton syndrome, supporting SPINK5 involvement in patients with EoE.^{129,130} SPINK5 is enriched in the esophageal epithelium and downregulated in biopsy specimens of patients with EoE.²⁸ SPINK5 is a multidomain proteinase inhibitor that consists of 15 potential serine proteinase inhibitory domains. Furin cleaves SPINK5 into fragments that contain 1 or more inhibitory domains.¹³¹ Each domain has different affinities to a variety of serine proteases. Therefore deficiency of SPINK5 in the esophagus can unleash uncontrolled proteolytic activity of numerous proteases. In support of its role in the esophagus, a WES analysis identified rare mutations in *SPINK5* in a cohort of patients with EoE,²⁸ and a candidate-gene approach linked 4 SNP sites in the *SPINK5* gene with EoE susceptibility in an EoE cohort compared with the nonatopic cohort.⁸⁵ The molecular pathways that are orchestrated by SPINK proteins in the esophagus merit further investigation.

SPINK7 is another SPINK member that has emerged as a key regulator of epithelial homeostasis in the esophagus.⁷⁰ SPINK7 is highly expressed by healthy esophageal epithelial cells and is decreased markedly in patients with EoE. Interestingly, SPINK5 and SPINK7 have distinct spatial and cellular expression patterns in the esophagus, suggesting that SPINK7 function in the esophagus is not completely redundant with SPINK5. The low percentage of identity between the active domains of SPINK5 and SPINK7 suggests that each inhibitor has different affinities to distinct repertoire of proteases, and therefore each inhibitor orchestrate unique pathways in the esophagus. Loss of SPINK7 is sufficient to induce epithelial barrier impairment and a marked production of proinflammatory and proallergic cytokines and chemokines by epithelial cells, including TSLP. In addition, loss of *SPINK7* and subsequent aberrant regulation of its downstream targets result in a defect in esophageal epithelial cell differentiation, induction of EMT, and production of an innate transcript signature that overlaps with processes seen in EoE-related allergic inflammation. Studies with SPINK7-deficient epithelial cells suggest that loss of SPINK7 might be upstream of loss of SPINK5, providing a mechanistic connection between these squamous epithelial enriched SPINK family members.^{70,105} SPINK7 mediates its function partially by inhibiting uPA, whereas other targets of SPINK7 are yet unknown. Preliminary restoration of SPINK7 has been demonstrated to have anti-EoE-like activity, at least *in vitro*.⁷⁰ Identification of SPINK7 downstream targets is important for a deeper understanding of the dysregulated pathways and future intervention strategies for EoE therapy.

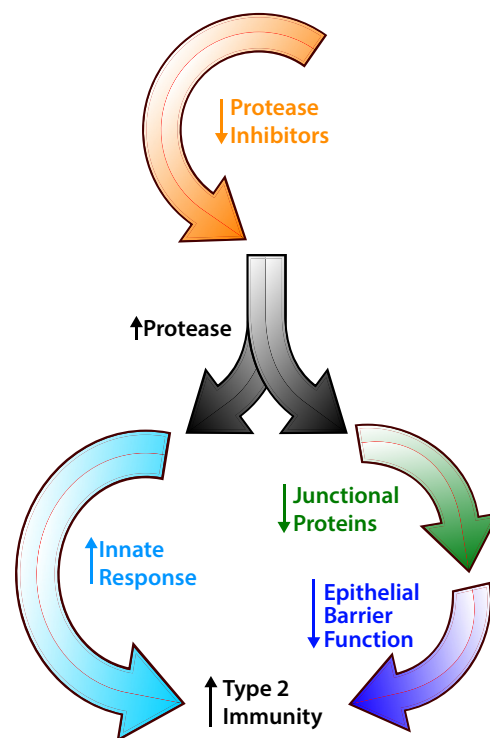


FIG 5. A schematic illustration of the potential role of the balance between proteases and protease inhibitors in the esophagus. Downregulation of protease inhibitors unleashes uncontrolled proteolytic activity. Proteolytic activation can lead to innate immune response by PAR2 or Toll-like receptor 4 activation, which in turn promote type 2 immunity. In addition, increased proteolytic activity can result in degradation of junctional proteins and an impaired epithelial barrier. As a result, antigen-presenting cells show increased access to external antigens and promote type 2 immunity.

The esophagus is also enriched with SERPINs from clade B of the superfamily of SERPINs (SERPINB), including *SERPINB2*, *SERPINB3*, *SERPINB4*, *SERPINB5*, *SERPINB11*, and *SERPINB13* (Fig 4). Expression of the majority of these SERPINs is altered in biopsy specimens of patients with EoE compared with control subjects (Fig 4). WES analysis of patients with EoE identified 2 rare mutations in *SERPINB3* (located on chromosome 18) in 3 unrelated patients.²⁸ SERPINBs are intracellular SERPINs that possibly protect cells from exogenous and endogenous protease-mediated injury. Interestingly, a novel FA locus had been identified in the SERPINB gene cluster at 18q21.3. This locus is associated with increased risk for FA with genome-wide significance.¹⁰⁴ One lead SNP, rs12964116, is located in an intron of *SERPINB7*, which is expressed in the upper layers of the epidermis, and loss-of-function mutations in this gene result in Nagashima-type palmoplantar keratosis syndrome, an autosomal recessive keratoderma associated with skin barrier impairment.¹³² Apart from the skin, *SERPINB7* is expressed in a few stratified squamous epithelia, including the esophagus. Its expression pattern suggests a potential role in the epithelial barrier integrity and function of the upper digestive tract, which might be relevant to the development of FA. The second SNP from this study, rs1243064, was correlated with *SERPINB10* expression in the blood.

Interestingly, although SERPINBs are emerging as potential drivers of both EoE and FA, distinct SERPINBs regulate these diseases.¹⁰⁴ These findings further demonstrate the similarities

between the 2 atopic conditions and can partly explain the high prevalence of FA in patients with EoE. Further investigation is needed to understand the fine tuning of these SERPINs and how SERPINs can potentially dictate the risk to acquire EoE, FA, or both. In addition, the roles of SERPINs and their targets are mostly unknown.

Another protein from the same superfamily is SERPINE1 (also known as PAI-1), which is induced in patients with EoE compared with control subjects. SERPINE1 is regulated by TGF- β 1 and has been shown to mediate TGF- β 1-induced fibrosis in esophageal epithelial cells *in vitro*.^{74,105,106}

To add further complexity, the esophageal epithelium is not the only source of proteases and protease inhibitors in patients with EoE. The presence of esophageal mast cells and basophils in inflamed esophageal epithelium in patients with EoE has been described in the past 2 decades. Mast cells and basophils contain preformed proteases that are stored in intracellular secretory granules and discharged upon activation. Mast cell degranulation is observed in patients with EoE compared with control subjects.¹³³ In addition, mast cell tryptase A and carboxypeptidase A expression is detected in patients with EoE. Tryptase regulates bronchoconstricting activities, antibacterial peptide activity, and protease-activated receptor 2 (PAR2) activation¹³⁴; chymase and mast cell carboxypeptidase A3 regulate the endothelin-1 pathway; and chymase and cathepsin G can degrade and eliminate cytokines.¹³⁴ These processes become germane in patients with EoE when these proteases are released into the environment deprived of counteracting protease inhibitors. Fig 4 summarizes different families of proteases and protease inhibitors that are enriched in the esophagus and are dysregulated in patients with EoE.

CONCLUSION

The role of proteases and their inhibitors is not limited to regulating epithelial barrier function but is also implicated directly in inducing type 2 immune responses. Proteases can elicit release of epithelium-derived T_H2 cytokines and chemokines by activating PAR2 and indirectly by promoting the release of fibrinogen cleavage products, which activate Toll-like receptor 4 signaling. Proteases can also cleave antimicrobial peptides and cytokines, such as IL-1 β , IL-18, IL-33, IL-36, and TGF- β 1, resulting in their activation.¹³⁵⁻¹³⁷

Notably, the IL-1 pathway is enriched in esophagus-specific genes,²⁸ suggesting that regulation of this pathway by proteases might be potentially critical in maintaining proper esophageal homeostasis. Data have already indicated aberrant regulation of the IL-1 pathway occurs in patients with EoE (eg, IL-33 overexpression), and identifying the responsible transcriptional and proteolytic mechanisms is underway; their importance for disease propagation certainly requires further investigation.

It is interesting to speculate that increased exposure to chemical proteases (eg, those found in detergents) might be a recently changed environmental exposure¹³⁸ that is linked with the EoE epidemic, and future research might prove an etiologic contribution. In addition, some allergens contain an exogenous source of proteases that directly activate PAR2 and induce type 2 immunity; therefore the expression of protease inhibitors by epithelial cells is critical for limiting the proteolytic activities of such allergens. The involvement of proteases in these processes has been shown in several allergic disorders, including asthma, AD, Netherton syndrome, and chronic rhinosinusitis with nasal polyps.^{128,139-141}

We have presented evidence that the epithelium is a primary driver of EoE pathogenesis. Genetic linkage of EoE susceptibility has uncovered the importance of epithelium-derived genes, such as *TSLP* and *CAPN14*. Resultant eosinophilia is likely secondary to epithelial activation by T cell–derived cytokines, which is most highlighted by IL-13 (with subsequent release of eosinophil active cytokines, such as eotaxin-3). The EoE epithelium is defective in its differentiation and dysregulates a number of key transcriptional programs, most notably reflected by loss of esophageal identity genes, as well as imbalanced expression of proteases and their inhibitors, and substantial evidence is emerging linking the SPINK family of protease inhibitors, especially SPINK7. As such, protease inhibitors might have therapeutic potential for the treatment of EoE. We provide an illustration of how proteolytic enzymes and their inhibitors can be involved in EoE, with a particular focus on the central involvement of the epithelium (Fig 5). Identifying the endogenous substrate repertoire of the proteases and their coupled inhibitors will facilitate our understanding of their roles in EoE pathobiology, and development of specific inhibitors might provide new strategies for the treatment of EoE. Given that the esophageal epithelium is reachable by topical delivery of drugs, as demonstrated by the utility of simply swallowing glucocorticoids designed for respiratory delivery to patients with asthma,¹⁴²⁻¹⁴⁸ the findings summarized in this article present new epithelium-based strategies to treat EoE.

We propose a previously unappreciated role for the esophagus as an anti-inflammatory sensing organ and that epithelial proteases/protease inhibitor imbalances unleash inflammatory responses, resulting in disorders, such as EoE.

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